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# ESTUDIOS FILOGENÉTICOS Y EVOLUTIVOS SOBRE "XANTHOPARMELIA" Y GÉNEROS RELACIONADOS (PARMELIACEAE, ASCOMYCOTA)

**MEMORIA PARA OPTAR AL GRADO DE DOCTOR**
**PRESENTADA POR**

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Bajo la dirección de los doctores

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# Estudios filogenéticos y evolutivos sobre *Xanthoparmelia* y géneros relacionados (Parmeliaceae, Ascomycota)

Guillermo Amo de Paz

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Informan:

Que la presente tesis doctoral titulada **"Estudios filogenéticos y evolutivos sobre *Xanthoparmelia* y géneros relacionados (Parmeliaceae, Ascomycota)"** ha sido realizada bajo su dirección por el Licenciado en Biología Guillermo Amo de Paz y, al estar concluida, autorizan su presentación para que sea juzgada por el tribunal correspondiente. Y para que así conste firman el presente informe en Madrid a 4 de Septiembre de 2012.


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*“La Géographie n'est autre chose que l'Histoire dans l'espace,*
*de même que l'Histoire est la Géographie dans le temps’’*

*La Geografía no es otra cosa que la Historia en el espacio,*
*así como la Historia es la Geografía en el tiempo*

Elisée Reclus (1830 – 1905)

## Resumen

El objetivo general de esta tesis ha sido profundizar en el conocimiento de la historia evolutiva del género *Xanthoparmelia*. Para ello se han estudiado los géneros próximos *Karoowia, Omphalodiella* y *Placoparmelia* mediante análisis filogenéticos usando marcadores moleculares de ADN y el estudio comparado de sus caracteres fenotípicos. Estos tres géneros pertenecen al mismo linaje evolutivo que las especies del género *Xanthoparmelia* y presentan, al igual que el resto de especies de este género, el polisacárido de pared tipo 'Xanthoparmelia liquenana' y un cuerpo vacuolar araquiforme en el interior de la ascospora. Los caracteres fenotípicos en los que se basó la descripción de los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia* se incluyen dentro de la variabilidad morfológica del género *Xanthoparmelia*, salvo el biotipo escuamuloso-peltado de *Omphalodiella*, lo que supone ampliar el rango de adaptaciones de este linaje. Como resultado del estudio los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia* se han sinonimizado con *Xanthoparmelia*.

Con el objetivo de estudiar las especies de amplia distribución del grupo '*Xanthoparmelia pulla*' se ha datado el origen de la diversificación de los líquenes parmelioides y se ha valorado si la distribución disyunta entre continentes es debida a vicarianza por deriva continental o a dispersión a larga distancia. Los resultados muestran que los líquenes parmelioides se originaron en torno al tránsito entre el Cretácico y el Terciario (60 Ma) y la radiación de los diferentes géneros se produjo entre el Oligoceno y el Plioceno, por lo que las distribuciones actuales a nivel de género concuerdan sólo con la dispersión a larga distancia, salvo en el caso de las disyunciones entre Sudamérica y Australia, en la que la edad de los géneros no permite descartar la vicarianza.

En el caso de las especies del grupo '*Xanthoparmelia pulla*' que presentan una amplia distribución, la filogenia no respalda el concepto fenotípico de estas especies, sino que muestra una correspondencia entre los diferentes linajes evolutivos, la distribución geográfica de los especímenes y los metabolitos secundarios medulares. Según los resultados del análisis filogeográfico, el grupo '*Xanthoparmelia pulla*' comenzó su radiación hace unos 11 Ma, con una alta probabilidad en Sudáfrica, lo que coincide con la aridificación de los ecosistemas en esta zona. Desde este territorio se ha extendido por fenómenos de dispersión a larga distancia a otros continentes, donde ha sufrido posteriores radiaciones. Desde Australia ha colonizado secundariamente Sudamérica también por dispersión a larga distancia.

## Introducción general

Los líquenes u hongos liquenizados son hongos que viven asociados con algas en lo que se considera una relación de mutuo beneficio para ambas partes (simbiosis). En esta relación el hongo forma una estructura estable, el talo, en cuyo interior viven las algas, que producen el alimento del que se nutre el hongo. Actualmente hay descritas unas 18.000 especies de estos hongos (Feuerer & Hawksworth, 2007) y los podemos encontrar en casi cualquier lugar, desde los desiertos helados de los polos hasta las zonas tropicales (Honegger, 2008).

El estudio de la diversidad de formas de vida de estos hongos se ha basado tradicionalmente en el estudio de sus caracteres morfológicos, anatómicos, de composición química o de las características ecológicas del medio en el que viven. Al ser organismos bastante simples en cuanto a estos caracteres, la información disponible tanto para la descripción de la diversidad de formas de vida que presentan, como para el esclarecimiento de las relaciones y procesos evolutivos que han conformado esta diversidad ha sido escasa, si los comparamos con otros organismos más complejos en cuanto a estructura.

Sin embargo, desde hace casi dos décadas, los marcadores moleculares de ADN nos han permitido identificar los linajes filogenéticos en los que han ido evolucionando los hongos liquenizados, hasta el punto de que sabemos que la adaptación de los hongos a esta forma de vida (la simbiosis con algas o liquenización) ha sido un proceso que ha ocurrido en más de una ocasión en la historia evolutiva de los hongos (Gargas *et al.*, 1995; James *et al.*, 2006).

Gracias a los marcadores moleculares de ADN se han podido construir robustas filogenias y se han conseguido grandes avances en el conocimiento de la historia evolutiva de estos organismos. Este conocimiento de la historia evolutiva de los hongos liquenizados se traducido también en cambios en su clasificación.

La gran mayoría de los hongos liquenizados (*ca.* 98%) pertenecen al Phylum Ascomycota. Dentro de este gran grupo de hongos, caracterizado por producir sus meiosporas dentro de unas células especializadas denominadas ascos, *ca.* 42% de sus especies presentan la liquenización como estrategia nutricional. De los 15 órdenes del Phylum Ascomycota que poseen alguna especie de hongo liquenizado, 10 de ellos pertenecen a la clase Lecanoromycetes, siendo el orden Lecanorales el mayor de ellos en cuanto a número de especies, con *ca.* 5500 especies (Honegger, 2008).

Dentro del orden Lecanorales hay descritas 20 familias (Lumbsch & Huhndorf, 2007). Una de las ellas, la familia Parmeliaceae, incluye *ca.* 2500 especies, y se caracteriza por presentar un apotecio de excípulo cupulado, ascos tipo 'Lecanora' y, en la mayoría de los casos, esporas hialinas (Crespo *et al.*, 2007). En base a los recientes estudios filogenéticos usando marcadores moleculares de ADN se han detectado 6 linajes monofiléticos principales dentro de la familia Parmeliaceae: son los grupos alectorioide, cetrarioide, hypogymnioide, letharioide, parmelioide y psiloparmelioide (Crespo *et al.*, 2007). Cada uno de estos linajes o clados monofiléticos agrupa en la mayoría de los casos a más de un género. De estos 6 linajes, el que presenta más diversidad de especies es el linaje parmelioide, con aproximadamente 1500 especies (Hale & DePriest, 1999, Crespo *et al.*, 2010).

Fenotípicamente los líquenes parmelioides se caracterizan por ser mayoritariamente foliáceos, con ricinas en la cara inferior, apotecios en forma de copa en posición laminar (no marginal) en la cara superior, ascos tipo 'Lecanora' y esporas hialinas (Crespo *et al.*, 2010). La delimitación de los géneros en el linaje parmelioide ha sido tradicionalmente muy controvertida, con algunos autores que reconocían sólo un género (*Parmelia s. lat.*) (Poelt & Vezda, 1981; Clauzade & Roux, 1985; Eriksson & Hawksworth, 1986; Llimona & Hladun, 2001) frente a otros liquenólogos que sugirieron agruparlas en unos 35 géneros (Hale, 1984; Elix, 1993; Hale & DePriest, 1999). Esta controversia fue debida fundamentalmente a que se dio poca importancia a la singularidad del ascoma y a la importancia básica que se atribuyó a los caracteres vegetativos morfológicos y químicos. Los recientes estudios filogenéticos han detectado 9 grandes linajes dentro del clado parmelioide en los que se reconocen 28 géneros y diversos linajes monofiléticos aun nos descritos como géneros. Estos estudios han ayudado a reevaluar los caracteres fenotípicos previamente usados para caracterizar los géneros ya descritos y han permitido la descripción de otros nuevos (Crespo & Cubero, 1998; Blanco *et al.*, 2004a; 2004b, 2005, 2006; Thell *et al.*, 2004; 2006; Divakar *et al.*, 2006, 2010a; Crespo *et al.*, 2007, 2010; Del Prado *et al.*, 2007). Los 9 clados o linajes reconocidos dentro de los líquenes parmelioides son los siguientes:

- Clado 'cetrelia' (18 especies). Un solo género incluido: *Cetrelia*

- Clado 'hypotrachyna' (243 especies). Géneros o grupos monofiléticos que incluye: *Cetrariastrum*, *Everniastrum*, *Hypotrachyna s. str.,* 'Hypotrachyna 1', 'Hypotrachyna 2' y *Parmelinopsis*.

- Clado ‘melanohalea’ (43 especies) Géneros o grupos monofiléticos que incluye: *Emodomelanelia*, *Melanelixia*, *Melanohalea*, ‘Melanelia 1’ y *Pleurosticta*.

- Clado ‘nipponoparmelia’ (4 especies) Un solo género incluido: *Nipponoparmelia*

- Clado ‘parmelia’ (102 especies) Géneros o grupos monofiléticos que incluye: *Parmelia s. str.*, Parmelia 1, *Relicina* y *Relicinopsis*.

- Clado ‘parmelina’ (82 especies). Géneros o grupos monofiléticos que incluye: *Bulbothrix s. tr.*, ‘Bulbothrix-Parmelinella’, *Myelochroa*, *Parmelina*, *Remototrachyna*.

- Clado ‘parmeliopsis’ (6 especies). Un género incluido: *Parmeliopsis*

- Clado ‘parmotrema’ (481 especies). Géneros o grupos monofiléticos incluidos: *Austroparmelina*, *Canoparmelia s.str.*, “Canoparmelia 1”, *Flavoparmelia*, *Flavopunctelia*, *Nesolechia*, *Parmotrema*, *Punctelia*.

- Clado ‘xanthoparmelia’ (819 especies) Géneros incluidos: *Karoowia, Omphalodiella* y *Xanthoparmelia* (Blanco *et al.*, 2004b; Thell *et al*., 2004, 2006; Crespo *et al.*, 2007).

La historia evolutiva del clado ‘xanthoparmelia’ es el objetivo principal de esta tesis doctoral. Por número de especies descritas se trata de uno de los mayores linajes de la familia Parmeliaceae. El género principal de este clado es *Xanthoparmelia* Hale, con *ca.* 800 especies. Fue descrito por Hale en 1974 para agrupar las especies saxícolas del género *Parmelia* que presentan ácido úsnico en el córtex superior, ricinas simples y carecen de cilios en el borde de los lóbulos (Hale, 1974). Posteriormente fue objeto de una extensa monografía en la que se estudiaban sus caracteres morfológicos, anatómicos y químicos, se incluía la descripción, tipificación y sinonimias de todas las especies conocidas hasta ese momento y se aportaba una clave para su identificación (Hale, 1990). *Xanthoparmelia* presenta su mayor de diversidad en las regiones áridas y semi-áridas del hemisferio sur, principalmente Sudáfrica y Australia, con un notable número de especies extendiéndose en el hemisferio Norte y en las regiones templadas de ambos hemisferios. Crecen sobre suelos y rocas silíceos y se diferencian fenotípicamente de otros parmelioides en que poseen el polisacárido de pared celular tipo ‘xanthoparmelia lichenana’, no presentan verdaderas pseudocifelas pero tienen un epicórtex porado y

muestran una considerable variación en la química cortical, incluyendo especies que presentan ácido úsnico, atranorina o que carecen de fenoles corticales (Blanco *et al.*, 2004b, 2006). Además todas las especies están caracterizadas por la presencia de un cuerpo vacuolar araquiforme (con forma de cacahuete) en las ascósporas (Del Prado *et al.*, 2007).

El género *Xanthoparmelia* resulta un buen ejemplo de los recientes cambios en la clasificación genérica de los líquenes, ya que los estudios moleculares, morfológicos y químicos concluyeron que, siguiendo un criterio filogenético, varios géneros previamente segregados debían incluirse en este único género. Por tanto se hicieron sinónimos de *Xanthoparmelia* géneros reconocidos previamente en base a su química cortical como *Neofuscelia* Essl. y *Paraparmelia* Elix & Johnst.; otros reconocidos en base a su biotipo como *Almbornia* Essl., *Chondropsis* Nyl. Ex Cromo. y *Xanthomaculina* Hale; o reconocidos por la presencia de "pseudocifelas" en la cara superior, como *Namakwa* Hale (Hawksworth & Crespo, 2002; Elix, 2003; Blanco *et al.,* 2004b; Thell *et al.,* 2006). Del mismo modo, en otros grupos de hongos liquenizados también se han revisado las clasificaciones genéricas previas que establecían diferencias en base a caracteres morfológicos vegetativos como la forma de crecimiento (*e.g.* Stenroos *et al.,* 2000; Tehler & Irestedt, 2007, Högnabba, 2006).

Los géneros *Omphalodiella* Hessen y *Placoparmelia* Hessen no habían sido estudiados en destalle con anterioridad a nuestro trabajo. Sin embargo por un análisis filogenético previo en el caso de *Omphalodiella* (Crespo *et al.,* 2007), o por sus características morfológicas, en el caso de *Placoparmelia*, se conocía su relación con el clado 'xanthoparmelia'. Estos géneros acomodan cada uno una sola y rara especie descrita a partir de especímenes que fueron recolectados en su día en la Patagonia argentina (Hessen, 1991, 1992) y su descripción como géneros independientes se basó en la forma de crecimiento del talo y la anatomía del apotecio.

*Omphalodiella patagonica* Hessen es un pequeño liquen peltado con apotecio aspicilioide. El tipo y el desarrollo del ascoma lo encuadran como un género morfológicamente inusual de la familia Parmeliaceae. La estructura del ascoma presenta una peculiar anatomía, teniendo un excípulo cupulado parmelioide fuertemente compacto e indiferenciado en capas. Además el excípulo propio aparece de forma prominente en la superficie del apotecio. *Omphalodiella* está también caracterizado por la ausencia de pseudocifelas y por esporas pequeñas con la pared fina (Hessen, 1991).

*Placoparmelia patagonica* Hessen es un liquen placoideo con apotecios inmersos que llegan a ser sésiles en su madurez. La estructura del ascoma en *Placoparmelia* es similar a la de *Omphalodiella* (Hessen, 1992). Junto a esta diferencia en el apotecio, *Placoparmelia patagonica* presenta otra peculiaridad, al carecer de córtex inferior y de verdaderas ricinas. En el resto de caracteres morfológicos, *P. patagonica* es muy similar a las especies placoideas de *Neofuscelia*, actualmente incluidas en el grupo *Xanthoparmelia squamarinata* (Elix, 1994). Debido a las similitudes en el ascoma y a su exclusiva distribución, Hessen formuló la hipótesis de que *Placoparmelia* y *Omphalodiella* podían representar un linaje característico dentro de los líquenes parmelioides que había evolucionado en Sudamérica (Hessen, 1992).

En el primer artículo de esta tesis doctoral se presenta el estudio de la posición filogenética de *Omphalodiella patagonica* y *Placoparmelia patagonica* según los marcadores moleculares de ADN y el análisis comparado de sus caracteres fenotípicos. Con estos datos se valora la clasificación de estos taxones.

*Karoowia* Hale es otro género insuficientemente estudiado previamente (Blanco *et al.*, 2004b, 2006; Crespo *et al.*, 2007; Thell *et al.*, 2004) y que incluye 19 especies (Elix, 1997, 1999, 2000). Fue descrito por Hale en 1989 para acomodar 16 especies subcrustosas similares a las especies del género *Xanthoparmelia* pero que diferían en tener rizoides en lugar de verdaderas rizinas y en producir conidios cilíndricos más largos (6 – 12 µm de longitud) en lugar de los cortos conidios bifusiformes a baciliformes (4 – 9 µm) presentes en *Xanthoparmelia* (Hale, 1989). Sin embargo, estudios moleculares recientes mostraron que dos especies de *Karoowia* estaban relacionadas con el clado 'xanthoparmelia' (Blanco *et al.*, 2004b; Thell *et al.*, 2004), aunque una de ellas, *Karoowia saxeti*, aparecía en un linaje que podría constituir el grupo hermano del clado 'xanthoparmelia' (Blanco *et al.*, 2004b, Crespo *et al*., 2007).

En el segundo capítulo de esta tesis doctoral se presenta la filogenia del género *Karoowia* con respecto al clado 'xanthoparmelia' y se realiza un análisis comparado de sus caracteres fenotípicos. Como en el caso anterior, se discute la clasificación de este género.

Al igual que existe una gran dificultad y controversia para reconocer los linajes o clados a los que se asigna el rango taxonómico de género debido a la escasez y plasticidad de los caracteres fenotípicos, es también difícil la delimitación y reconocimiento en el rango de especie, unidad básica de la taxonomía. A este respecto los marcadores moleculares han supuesto también un gran avance, con numerosos estudios que han

ayudado a la delimitación o han puesto de manifiesto la existencia de una diversidad críptica que no se refleja en los caracteres fenotípicos. También se ha encontrado el caso contrario, en el que diferencias fenotípicas que se habían considerado como caracteres válidos para diferenciar especies en realidad no lo eran y deben ser considerados variabilidad intraespecífica (Crespo & Pérez-Ortega, 2009; Lumbsch & Leavitt, 2011). Recientemente, en el género *Xanthoparmelia* se ha llevado a cabo un profundo estudio de poblaciones en un conjunto de 19 especies que habitan en el Oeste de EEUU. En este estudio han sido secuenciados 6 marcadores moleculares con el objetivo de delimitar especies y en él se ha puesto de manifiesto la dificultad de la caracterización fenotípica de los linajes evolutivos (Leavitt *et al.*, 2011a,b).

Unido a esta dificultad para delimitar especies basándose en los caracteres fenotípicos hay una dificultad operativa derivada del hecho de que numerosos líquenes presentan grandes áreas de distribución. Es decir, es frecuente que en hongos, tanto liquenizados como no liquenizados, existan especies con amplias distribuciones que abarcan varios continentes, o incluso especies cosmopolitas (Taylor *et al.*, 2006; Galloway, 2008). Este hecho ha llevado a extender para estas especies de amplia distribución, el concepto bien arraigado en microbiología de que "todo está en todos los sitios", haciendo referencia a que la presencia o ausencia de una especie está determinada por factores ecológicos y no por eventos de su historia evolutiva. El concepto se fundamenta biológicamente en que la cantidad de individuos de las poblaciones es tan grande y la dispersión tan constante, que pueden alcanzar cualquier localidad geográfica (Finlay, 2002; Fenchel & Finlay, 2004). Otros taxones de hongos presentan sin embargo distribuciones más restringidas, llegando en ciertos hongos liquenizados a presentar un notable grado de endemismo, como ocurre en Nueva Zelanda, donde el 23% de las especies presentes sólo habitan allí, o la Antártida continental, con áreas donde el grado de endemismo llega al 50% (Galloway, 2008). En este aspecto también los marcadores moleculares están arrojando nuevos datos que nos hacen pensar que algunas especies de hongos que se creía que presentaban una amplia distribución (siguiendo una taxonomía basada en caracteres fenotípicos), tienen en realidad una distribución más restringida y que bajo esas grandes áreas de distribución existe una diversidad críptica con unas áreas de distribución más pequeñas (Taylor *et al.*, 2006). También en hongos liquenizados varios estudios han demostrado que diversas especies consideradas de amplia distribución presentan diferentes linajes evolutivos restringidos a una parte de esas grandes áreas (Arguello *et al*., 2007; Elix *et al*., 2009; Divakar *et al.*, 2010b; Otálora *et al.*, 2010; Sérusiaux *et al.*, 2011). En otros trabajos, por el contrario, las especies estudiadas presentaban linajes que corroboran la amplia distribución (Myllys *et al.*, 2003; Molina *et al.*, 2004; Buschbom, 2007).

En dos especies del género *Xanthoparmelia* se ha encontrado un patrón de distribución de los linajes evolutivos que restringe el área de distribución de las especies y hace aflorar diversidad no esperada. Estas dos especies están distribuidas en Norte América y Australia y el estudio de los marcadores moleculares ha puesto de manifiesto que en cada continente los especímenes pertenecen a diferentes linajes (Thell *et al.*, 2009; Hodkinson & Lendemer, 2011).

El hecho de que para algunos de los hongos liquenizados exista una correspondencia entre los linajes evolutivos y sus áreas de distribución hace que la biogeografía de estos organismos cobre aún mayor importancia a la hora de estudiar su historia evolutiva. Los postulados tradicionales para explicar las áreas de distribución de los hongos liquenizados, al igual que en el resto de los organismos, han tenido en cuenta dos fenómenos principales: la vicarianza (un área primaria de distribución continua que se fragmenta dejando poblaciones aisladas que evolucionarán independientemente) y la dispersión a larga distancia. La filogeografía integra estos postulados biogeográficos con los estudios de genética de poblaciones y de las relaciones filogenéticas entre los grupos (Avise, 2000).

Un dato importante en los estudios filogeográficos es conocer o tratar de estimar las edades de los linajes evolutivos. Al estimar la edad de un linaje que presenta un área de distribución disyunta podemos dilucidar (i) si esta distribución se debe a la fragmentación de un área continua original (ejemplos: áreas fragmentadas por deriva continental, zonas afectadas por cambios climáticos como glaciaciones, desertizaciones, etc.) o (ii) si por el contrario esta distribución se debe a la dispersión a larga distancia desde una de las poblaciones a la otra (Werth, 2011). Mientras que en animales y plantas, en los que se cuenta con un buen registro fósil, las edades de divergencia de los grandes linajes están bien establecidos, en el caso de los hongos el escaso registro fósil ha dificultado la estimación de las edades de diversificación de los principales linajes. Las recientes estimaciones de datación para el origen de los hongos, basadas en la reinterpretación de la morfología de los fósiles y en la reevaluación de los estudios de datación, sugieren que se produjo hace entre unos 760 y 1060 Ma, mientras que la separación entre Ascomycota y Basidiomycota se produjo entre los 500 – 600 Ma (Berbee & Taylor, 1993, 2001, 2010; Heckman *et al.*, 2001; Padovan *et al.*, 2005; Taylor & Berbee, 2006; Lücking *et al.*, 2009;). Para la clase Lecanoromycetes se baraja una estimación de su origen en 280 – 320 Ma (Lücking *et al.*, 2009).

Algunos trabajos han llevado a cabo estimaciones de la edad en diferentes grupos de hongos liquenizados. Para ello se han usado diferentes técnicas de calibración, desde las

relaciones con cambios climáticos históricos (Printzen & Lumbsch, 2000), la emergencia de islas volcánicas (Sérusiaux *et al.*, 2011) o el uso de tasas de mutación calculadas para otros grupos de hongos a través del registro fósil (Otálora *et al.*, 2010; Sérusiaux *et al*., 2011). En nuestro caso, la estimación del origen y diversificación de los principales clados parmelioides se basa en la calibración de la filogenia a través de inferencias a partir del registro fósil. Dado que no se conocen fósiles o estimaciones de edad para el género *Xanthoparmelia* pero que disponemos ya de buenas filogenias que abarcan desde la clase Lecanoromycetes (Lumbsch *et al.*, 2007) hasta los líquenes parmelioides (Crespo *et al.,* 2010), en el capítulo 3 se presenta un análisis de estimación del tiempo de divergencia para los linajes de los líquenes parmelioides. Este paso previo nos permite disponer de una estimación de la edad de origen y diversificación del género *Xanthoparmelia*. Para la calibración de la filogenia de parmelioides se parte de la edad estimada de separación entre Lecanoromycetes y Chaethothyriomycetidae (Lücking *et al*., 2009) y se han usado dos puntos de calibración interna de la filogenia estimados a partir del registro fósil. Los fósiles empleados en esta calibración interna de la filogenia son una especie del género *Alectoria* que fue descrita en ambar del Mar Báltico (35 – 40 Ma) (Mägdefrau, 1954) y una especie del género *Parmelia* que fue descrita en ambar dominicano (15 – 45 Ma) (Poinar *et al.*, 2000).

Una vez que nuestro propio trabajo nos ha permitido disponer de una estimación de la edad para los líquenes parmelioides y sus principales clados, en el capítulo 4 abordamos un estudio filogeográfico de un grupo de especies del género *Xanthoparmelia* que presenta una amplia distribución abarcando varios continentes de ambos hemisferios. Para este trabajo se eligió el grupo '*Xanthoparmelia pulla*', que constituye un linaje interno del género *Xanthoparmelia.* En él existen además diversas especies descritas fenotípicamente que presentan una distribución disyunta entre varios continentes y en ambos hemisferios (Figura 1). Al igual que en el resto de grupos del género *Xanthoparmelia*, la delimitación de especies dentro del grupo '*X. pulla*' está actualmente basada en una combinación de caracteres fenotípicos (forma de los lóbulos, adherencia al sustrato y presencia de propágulos vegetativos, así como el color de la cara inferior o variaciones de la composición química de metabolitos secundarios medulares).

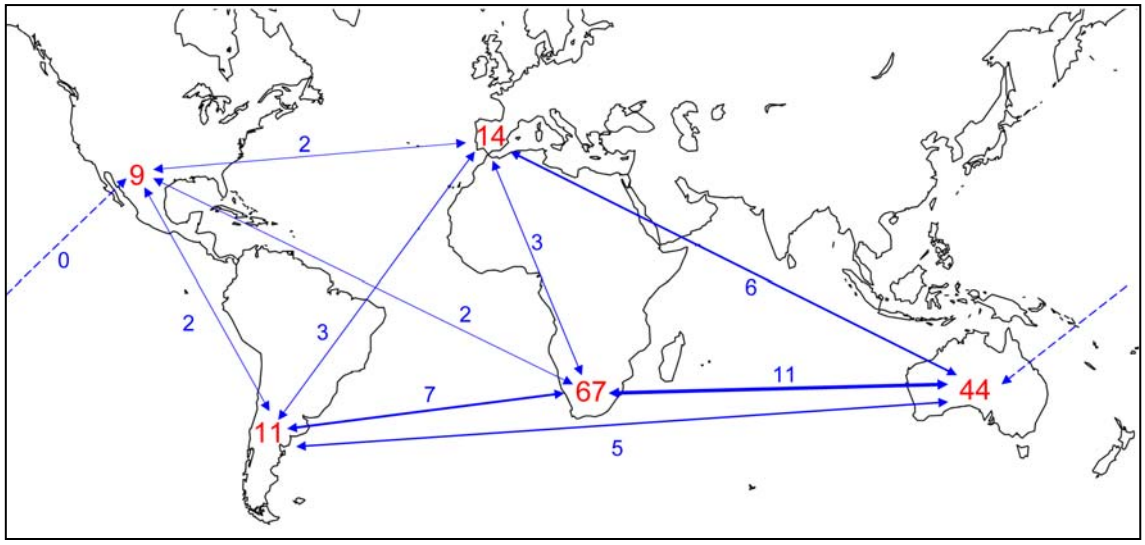


Figura 1. Riqueza de especies en el grupo '*Xanthoparmelia pulla*' en las cinco áreas estudiadas (en rojo). En color azul aparecen el número de especies compartidas entre dos territorios.

En las clasificaciones previas el grupo '*Xanthoparmelia pulla*' había sido clasificado dentro del género *Neofuscelia*. Este género fue descrito basándose en diferencias en la química cortical como la presencia de pigmentos melanoides y ausencia de ácido úsnico o atranorina, característicos de la mayoría de las especies de *Xanthoparmelia* (Esslinger, 1978, 2000). En un posterior estudio filogenético se comprobó que el género *Neofuscelia* era polifilético y fue sinonimizado con *Xanthoparmelia* (Blanco *et al.*, 2004b). Uno de los linajes en los que se fragmenta el género *Neofuscelia* es el grupo '*X. pulla*'.

Además de conocer la edad de los linajes e inferir los procesos que explican su distribución actual, en el capítulo 4 se presenta una reconstrucción del área ancestral de distribución para el grupo '*X. pulla*'.

## Objetivos

El objetivo general de la tesis ha sido profundizar en el conocimiento de la historia evolutiva del género *Xanthoparmelia*. Para ello se han planteado los siguientes objetivos específicos:

- Caracterizar el género *Xanthoparmelia* estudiando la filogenia de los géneros próximos *Omphalodiella, Placoparmelia* y *Karoowia* y realizar un estudio comparado de sus caracteres fenotípicos (Capítulos 1 y 2).

- Datar el origen y la diversificación de los líquenes parmelioides, grupo evolutivo al cual pertenece el género *Xanthoparmelia*. Con la estimación de las edades de los clados (1) se determinarán los procesos que explican las distribuciones geográficas actuales de los géneros y (2) se relacionará el periodo en que aparecieron y se diversificaron los diferentes linajes parmelioides con el paleoclima general del planeta (Capítulo 3).

- Explorar si existe un patrón filogeográfico en la radiación evolutiva del grupo '*Xanthoparmelia pull*a', que forma uno de los linajes internos del género *Xanthoparmelia* y presenta una amplia distribución que abarca varios continentes y ambos hemisferios, con varias especies de distribución disyunta entre continentes (Capítulo 4).

# Los géneros *Omphalodiella* y *Placoparmelia,* descritos en base a diferencias morfológicas, pertenecen a *Xanthoparmelia* (Parmeliaceae)

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## Resumen

Los géneros monotípicos *Omphalodiella* y *Placoparmelia* fueron descritos a partir de recolecciones de la Patagonia (Argentina). *Placoparmelia* está caracterizado por ser una especie placoidea, morfológicamente similar a algunas especies marrones y subcrustáceas de *Xanthoparmelia*, mientras que *Omphalodiella* es un liquen escuamuloso-peltado. El análisis de las secuencias de ADN ribosomal sitúan estos géneros en los líquenes parmelioides, dentro del clado 'xanthoparmelia'. La filogenia no respalda la hipótesis de que ambos géneros constituyan un linaje evolutivo propio de Sudamérica, hipótesis basada en la singularidad de sus apotecios. Ambos géneros presentan similitudes en los estadíos iniciales de su ascoma, al ser aspicilioides y compactos, pero en estado maduro, estas diferencias se atenúan. Un análisis detallado de la cara inferior de *Placoparmelia* muestra que el córtex inferior y las ricinas se encuentran muy reducidas, pero aparecen como estructuras vestigiales. En el córtex superior de *Omphalodiella* se observan pequeños poros, un carácter constante en *Xanthoparmelia*. El biotipo escuamuloso-peltado de *Omphalodiella* es una singularidad dentro del género *Xanthoparmelia* y constituye un aumento de la diversidad de adaptaciones morfológicas que presenta este linaje. La posición filogenética dentro de *Xanthoparmelia* de estos dos géneros es congruente con la presencia del polisacárido de pared celular tipo Xanthoparmelia liquenana y con el cuerpo vacuolar araquiforme de las ascósporas, dos caracteres propios del género *Xanthoparmelia*. Por tanto proponemos la sinonimia de estos dos géneros en *Xanthoparmelia* y la combinación de las dos especies de estos géneros con los nombres de *Xanthoparmelia patagonica* comb. nov.y *X. peltata* comb. nov.

# The morphologically deviating genera *Omphalodiella* and *Placoparmelia* belong to *Xanthoparmelia* (Parmeliaceae)

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**Abstract.** The monotypic genera *Omphalodiella* and *Placoparmelia* were described from Patagonia (Argentina). The latter is characterized by placodioid species, morphologically similar to some subcrustose, brown *Xanthoparmelia* species, whereas *Omphalodiella* is a peltate lichen. Analyses of ribosomal DNA sequences supported their placement in the parmelioid clade. Both genera are nested within *Xanthoparmelia*. Affinites to the latter genus are congruent with the presence of *Xanthoparmelia*-type lichenan in the hyphal cell walls and an arachiform vacuolar body in the ascospores, two key characters typical of *Xanthoparmelia*. Consequently we propose to place the generic names in synonymy with *Xanthoparmelia* and transfer the two species to the latter genus as *X. patagonica* and *X. peltata* comb. nov.

**Keywords.** Molecular phylogeny, Parmelioid genera, taxonomy, lichenan.

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Generic circumscriptions in lichen-forming fungi have changed dramatically since the late 1960s (Nimis 1998) with new genera erected for morphologically deviating groups of species in different families such as Physciaceae (Esslinger 1978, 1986; Lohtander et al. 2000; Poelt 1966) and Parmeliaceae (DePriest 1999; Elix 1993; Hale 1984; Rambold & Triebel 1999). The latter represents one of the largest families of lichenized fungi and the largest clade within this family, the parmelioid lichens, includes approximately 1500 known taxa (Crespo et al. 2007; Hale & DePriest 1999). Within the parmelioid lichens, morphological and chemical characters have generally been used to segregate genera and as a consequence, the acceptance of the genera segregated without using ascomatal characters has not been uniform (Clauzade & Roux 1985, Eriksson & Hawksworth 1986, Llimona & Hladun 2001; Poelt & Vězda 1981). Recent molecular studies have helped to identify monophyletic clades in the parmelioid lichens and prompted the re-evaluation of phenotypic characters previously used to circumscribe the genera (Blanco et al. 2004a, 2004b, 2005, 2006; Crespo & Cubero 1998; Crespo et al. 2007, 2010; Del Prado et al. 2007; Divakar et al. 2006, 2010; Thell et al. 2004; Thell et al. 2006). One of the

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major clades of parmelioid lichens is the *Xanthoparmelia*-clade (Blanco et al. 2006; Lumbsch et al. 2008). This clade includes more than 800 species, with centers of diversity in arid to semi-arid regions of the southern Hemisphere, but with several species extending into temperate regions. The species occur on siliceous rocks or soil. Species in this clade contain the polysaccharide *Xanthoparmelia*-type lichenan in the cell walls, lack true pseudocyphellae, have a pored epicortex, and show considerable variation in cortical chemistry, including species containing usnic acid, atranorin or lacking cortical phenols (Blanco et al. 2004b, 2006). Furthermore all species are characterized by the presence of an arachiform vacuolar body in the ascospores (Del Prado et al. 2007).

The *Xanthoparmelia*-clade provides a pertinent example of the changes in the generic classification of parmelioid lichens. Several recent publications on this clade have resulted in the incorporation of a number of previously recognized genera within *Xanthoparmelia*. Thus *Almbornia* Essl., *Chondropsis* Nyl. ex Cromb., *Namakwa* Hale, *Neofuscelia* Essl., *Paraparmelia* Elix & J. Johnst., and *Xanthomaculina* Hale have been synonymized with *Xanthoparmelia* (Blanco et al. 2004b; Elix 2003; Hawksworth & Crespo 2002; Thell et al. 2006). The *Xanthoparmelia*-clade is well supported as monophyletic, but within this clade, no robust clades were found. This may reflect a rapid adaptive radiation in this clade following a shift towards drier habitats at the base of the *Xanthoparmelia*-clade (Lumbsch et al. 2008). This habitat shift presumably led to different morphological adaptations (growth form, rhizine structure, morphology of apothecia, etc) whose significance has been overestimated in previous classifications.

Two genera that have not been studied in detail previously are *Omphalodiella* Henssen and *Placoparmelia* Henssen. These genera accommodate each a single and rarely collected species occurring in Patagonia (Argentina; Henssen 1991, 1992) and were circumscribed on the basis of their apothecial anatomy and growth forms.

*Omphalodiella patagonica* Henssen is small peltate lichen with aspicilioid apothecia. The ascus-type and ascomatal development placed this morphologically unusual genus in Parmeliaceae. The ascomatal structure presents a peculiar anatomy, having a parmelioid cupulate excipulum (subhymenium) strongly compact. *Omphalodiella* was characterized by the lack of pseudocyphellae and the small, thin-walled ascospores (Henssen 1991).

*Placoparmelia patagonica* Henssen is a placodioid lichen with immersed apothecia that become sessile at maturity. The ascomatal structure in *Placoparmelia* is similar to that of *Omphalodiella* (cupulate excipulum strongly compact; Henssen 1992) and was interpreted as being different to that observed in *''Neofuscelia''*. Further, unlike *''Neofuscelia''*, *Placoparmelia patagonica* lacks a lower cortex. Morphologically, *Placoparmelia* is very similar to the placodioid *''Neofuscelia''* spp. currently included in the *Xanthoparmelia squamariata* group (Elix 1994). Henssen (1992) speculated that *Placoparmelia* and *Omphalodiella* may represent a distinct lineage within the parmelioid lichens, which evolved in South America.

The aim of this study was to investigate the phylogenetic affinities of the genera *Placoparmelia* and *Omphalodiella* within Parmeliaceae and to determine their relationships with the *Xanthoparmelia*-clade using molecular markers. On the basis of the phylogenetic results we studied the morphological and chemical characters of these two genera to see whether their key characters were consistent with the phylogenetic placement as inferred from molecular data.

## Material and Methods

***Molecular analyses.*** We assembled a matrix composed of nuITS rDNA, nuLSU rDNA and mtSSU rDNA sequences representing 52 specimens of *Xanthoparmelia* retrieved from previous studies (Blanco et al. 2004b, 2006; Crespo et al. 2007), including sequences of the three genes of *Omphalodiella patagonica* (Argentina, Río Negro, *Lumbsch 11036a*, DQ980021, DQ923670, DQ923643). The following sequences were newly obtained for this study: nuLSU rDNA of *Placoparmelia patagonica* (Argentina, Santa Cruz, *Hessen & Vobis*, *Herbarium* no. *LD 1082542*, —, GU461304, —) and sequences of the nu ITS rDNA, nuLSU rDNA and mtSSU rDNA for *Xanthoparmelia*

aff. *caparidensis* (South Africa, Cape Region, *Crespo, Divakar, Hawksworth, Amo de Paz & Lumbsch, Herbarium* no. *MAF 16113*, GU461305, GU461303, GU461302). *Placoparmelia patagonica* is only known from the type and repeated attempts by Maria Ines Messuti (Bariloche) to collect fresh material at the type locality were unsuccessful. Despite repeated attempts, we were only able to obtain sequences of one molecular marker from the type specimen collected in 1973. We also included sequences of seven species of parmelioid lichens outside of the *Xanthoparmelia*-clade that were downloaded from the GenBank and used as outgroup based on previous phylogenetic studies (Crespo et al. 2007, Lumbsch et al. 2008). **Appendix 1** lists the GenBank accession numbers, locality, and voucher information of the sampled exemplars.

Frozen lobes of specimens were crushed with sterile glass pestles. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions and modifications as detailed elsewhere (Crespo et al. 2001). The following primers were used: for nuITS rDNA: and ITS1-LM (Myllys et al. 1999) and ITS2-KL (Lohtander et al. 1998); for nuLSU rDNA: LR0R and LR5 (Vilgalys & Hester 1990); and for mtSSU: mrSSU1 and mrSSU3R (Zoller et al. 1999).

For each amplification we used a reaction mixture of 50 µL, containing: 5 µL of 10× DNA polymerase buffer (Biotools) ($MgCl_2$ 2mM, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1mM EDTA and 0.1% Triton X-100), 2.5 µL of each primer, 1.25 µL of DNA polymerase (1U/µL), 1 µL of dinucleotide triphosphate (dNTPs) containing 10 mM of each base (Biotools), 10 µL of DNA (third elution of DNA extraction) and 27.75 µL $dH_2O$. Amplifications were carried out in an automatic thermocycler (Techne Progene 3000). Programs were performed with the following steps: Step 1: initial denaturation at 94°C for 5 min. Step 2: 35 cycles of: 94°C for 1 min, 60°C (nuITS rDNA), 58°C (nuLSU rDNA) or 56°C (mtSSU rDNA) for 1 min, and 72°C for 1.5 min. Step 3: final extension at 72°C for 5 min. We used a DNA Purification Kit (Flavorgen) to clean the PCR products. The cleaned PCR products were sequenced with the same primers using the ABI Prism Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) with the following program: initial denaturation at 94°C for 3 min followed by 25 cycles of three steps (96°C for 10s, 50°C for 5s and 60°C for 4 min). Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems). Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually edited. The sequences were aligned separately for each data set using Muscle 3.6 (Edgar 2004) and ambiguously aligned positions were removed manually.

We performed phylogenetic analyses using maximum parsimony (MP) and a Bayesian approach. To test for potential conflict, parsimony bootstrap analyses were performed on matrices corresponding to the individual loci, and 75% bootstrap consensus trees were examined for conflict (Lutzoni et al. 2004). MP analyses were performed using the program PAUP* 4.0b (Swofford 1993) with random additions and characters unordered and equally weighted. MulTrees option and branch swapping using TBR was in effect. Bootstrap analyses (Felsenstein 1985) were performed with 2,000 pseudoreplicates of random addition sequences. To assess homoplasy levels, CI, RI and RC index were calculated from each parsimony search.

A Bayesian analysis (B/MCMC) was performed using MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001) using the GTR + I + G model (Rodríguez et al. 1990). The data set were partitioned into three parts including nu ITS, nu LSU and mt SSU. Each partition was allowed to have its own parameter values (Nylander et al. 2004). No molecular clock was assumed. Heating of chains was set to 0.2. Posterior probabilities were approximated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. A tree was sampled every $1000^{th}$ generation from a total of 5 million generations. The first 3000 generations were discarded as burn in. We used AWTY (Nylander et al. 2007) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. Of the remaining 4997 trees, a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades with bootstrap support equal

or above 70% under MP and posterior probabilities ≥ 0.95 in the Bayesian analysis were considered as strongly supported. Phylogenetic trees were visualized using the program Treeview (Page 1996).

To evaluate whether our data set allows rejecting a placement of *Omphalodiella* and/or *Placoparmelia* outside *Xanthoparmelia*, we employed alternative hypothesis testing. ML trees inferred from unconstrained and constraint searches were obtained using Garli 0.98 (Zwickl 2006). For the hypothesis testing, we used two different methods: i) Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 2001) and ii) expected likelihood weight (ELW) test (Strimmer & Rambaut 2002). The SH and ELW test were performed using Tree-PUZZLE 5.2 (Schmidt et al. 2002) with the combined data set, comparing the best tree agreeing with the null hypotheses, and the unconstrained ML tree.

***Scanning-electron microscopy (SEM).*** Lichen thalli were washed in tap water. Thereafter they were dehydrated (Samson et al. 1979). Critical point dried specimens were mounted on stubs and sputter-coated with gold. The specimens were examined in a Jeol JSM 6400 SEM to observe the epicortex structure and lower surface in *Omphalodiella patagonica* and *Placoparmelia patagonica.*

***Cell-wall polysaccharide analysis.*** The presence of lichenan-types in the hyphal cell walls was determined using histochemical methods (Nash et al. 1990; Common 1991). Thallus cross-sections were cleared using Chlorox after wetting with a liquid detergent solution. Subsequently they were transferred to a series of iodine solutions (0.15, 1.5 and 20% IKI and Melzer's). At higher concentrations lichenan forms a red-brown complex, while isolichenan forms a blue complex in 0.15% IKI. Observations were made under Leica S6E dissecting microscope.

## Results and Discussion

Four new sequences were generated for this study (**Appendix 1**). The MP bootstrap analysis did not identify any conflicts (i.e. well supported differences in the topology) among the single-gene data sets; hence a multi-gene data set was analyzed. A matrix of 1972 unambiguously aligned nucleotide position characters with 545 positions in the nuITS, 559 positions in the mtSSU and 868 positions in the nuLSU data set was used for the analyses. The number of constant characters was 1579.

MP analysis of the combined data set, comprising 311 potential parsimony informative characters yielded 1152 most parsimonious trees, 1042 steps long (CI = 0.500, RI = 0.737, RC = 0.369). In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following values averaged for the three partitions (± standard deviation): base frequencies $\pi(A) = 0.264$ (± 0.0001), $\pi(C) = 0.218$ (± 0.0001), $\pi(G) = 0.264$ (± 0.0001), $\pi(T) = 0.254$ (± 0.0001), $\ln L = -8484.75$ (± 0.913), and the gamma shape parameter $\alpha = 0.591$ (± 0.0007). The topology of the trees from the MP (strict consensus tree) and Bayesian analyses did not show any conflict and hence only the Bayesian tree is shown here (**Fig. 1**), with MP bootstrap values above 70% as well as posterior probabilities equal or above ≥ 0.95 indicated.

In the phylogenetic tree inferred from the combined data set (**Fig. 1**), *Placoparmelia* and *Omphalodiella* are nested within the *Xanthoparmelia*-clade. *Omphalodiella patagonica* is sister to a clade including *Xanthoparmelia azaniensis* and *X. ovealmbornii*. This relationship is supported under MP and B/MCMC optimization criteria. *Placoparmelia patagonica* forms an unresolved relationship with two clades including taxa previously included in *Neofuscelia*, however, one of the clades lacks support. The relationship of *Placoparmelia* also lacks support and hence the placement of *P. patagonica* within *Xanthoparmelia* remains unclear but we tested the alternative hypothesis (e. g. *P. patagonica* sister of *Xanthoparmelia*), which was rejected in most cases (**Table 1**). The phylogeny of the remaining taxa in the *Xanthoparmelia*-clade has been discussed previously (Blanco et al. 2004b, Blanco et al. 2006).

Alternative hypothesis tests show that a placement of *Placoparmelia* and *Omphalodiella* outside of *Xanthoparmelia* can be significantly rejected (**Table 1**). Further, the molecular data do not support the hypothesis that *Placoparmelia* and *Omphalodiella* form an independent evolutionary lineage within the Parmelioid-clade, that would have originated in South America (Henssen 1992).

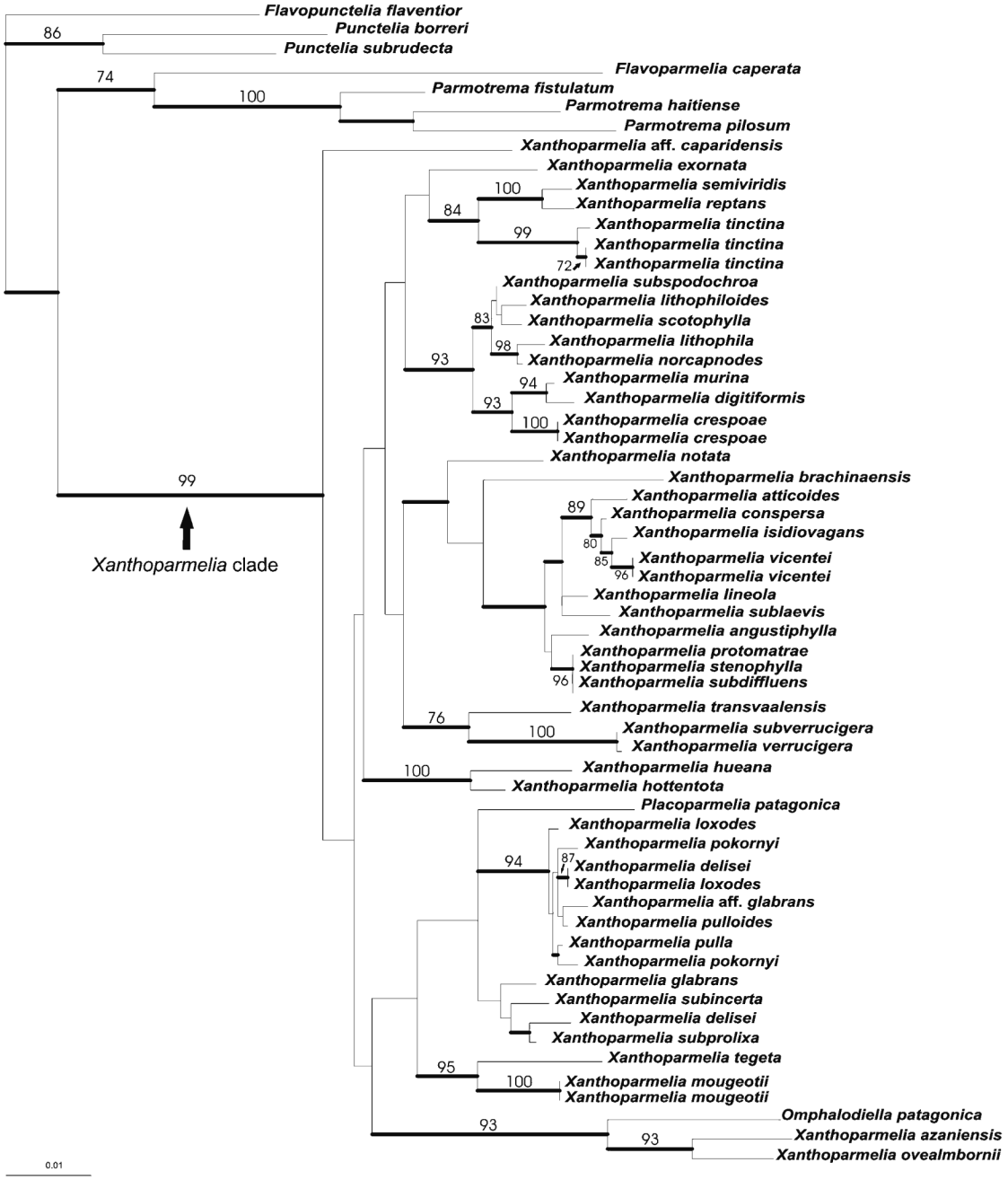


**Figure 1.** Phylogenetic tree showing the phylogenetic placement of *Omphalodiella* and *Placoparmelia* in the *Xanthoparmelia*-clade. This is a maximum-likelihood tree inferred from a combined data set of nuITS, nuLSU, and mtSSU rDNA sequences. Bootstrap values equal and above 70% under MP indicated at branches. Branches with posterior probabilities equal or above 0.95 in bold.

*Placoparmelia patagonica* is an effigurate-placodioid lichen with small lobes (0.2–0.8 mm broad) marginally with an areolate center (**Fig. 2B**). Apothecia are immersed when young (**Fig. 2B**) becoming sessile at maturity. The mature apothecia have the typical parmelioid hyaline subhymenium (**Fig. 2F**). This layer is more difficult to see in young apothecia and at the rim of the mature apothecia, since the rim is compact and thin (**Fig. 2F**). The mature ascospores of *P. patagonica* have an

**Table 1.** Results of the alternative hypothesis testing analyses. Probabilities of null hypotheses. * probabilities significant at < 0.05 and,** < 0.001.

| | *Probability* | |
|---|---|---|
| ***Null hypothesis*** | **p-SH** | **c-ELW** |
| *Omphalodiella* outside of *Xanthoparmelia* | 0.01* | 0.0006** |
| *Placoparmelia* outside of *Xanthoparmelia* | 0.032* | 0.016* |
| *Placoparmelia* sister to *Xanthoparmelia* | 0.046* | 0.02* |
| *Placoparmelia* sister to *Omphalodiella* | 0.014* | 0.001* |

arachiform vacuolar body, as found in other taxa of the *Xanthoparmelia*-clade (**Fig. 2D**). The epicortex is pored (Henssen 1992) and the cell-wall polysaccharide was found to be *Xanthoparmelia*-type lichenan. The lower surface of this strongly adnate species is very thin and consists of agglutinated, prosoplectenchymatous hyphae (**Fig. 2H**). Some hyphal bundles (small rhizines) occur at the margin of the small lobes (**Fig. 2H**).

*Omphalodiella patagonica* is a small peltate lichen, consisting of rounded squamules up to 2 mm in diam. (**Fig. 2A**) with numerous small, aspicilioid apothecia, up to 0.3 mm in diam. A hyaline subhymenium typically found in other parmelioid lichens is visible in center of the mature apothecia (**Fig. 2E**). The ascospores of *O. patagonica* also have an arachiform vacuolar body (**Fig. 2C**). This species has a pored epicortex (**Fig. 2G**) and the cell-wall polysaccharide analysis shows that it contains *Xanthoparmelia*-type lichenan.

*Omphalodiella patagonica* and *Placoparmelia patagonic*a were described as independent genera based on the differences in their ascoma anatomy and their growth forms (Henssen 1991, 1992). However, our molecular analyses show that both genera are nested within *Xanthoparmelia* and that they share with other *Xanthoparmelia* species key characters, such as polysaccharide- and ascospore-types, that have been shown to be phylogenetically informative within parmelioid lichens (Blanco et al. 2006; Crespo et al. 2007; Del Prado et al. 2007).

This study provides another example for the extreme variability in growth form in several, unrelated clades of lichen-forming fungi. There is a growing body of evidence that suggests that the growth form of lichens exhibit a high degree of plasticity (Arup & Grube 1998, 2000; Blanco et al. 2004b; Gaya et al. 2003; Grube & Arup 2001; Högnabba 2006; Søchting & Lutzoni 2003; Tehler & Irestedt 2007). These results also confirm that vegetative characters are poor phylogenetic discriminators, probably due to the lack of specialization of cell-types and the lack of true tissue in fungi (Jahns & Ott 1994). The morphological divergence in thalline morphology is most probably caused by adaptations to particular ecological factors (Grube & Hawksworth 2007). Peltate thalli, as those found in *Omphalodiella*, are common among lichens growing in exposed habitats on siliceous rocks and include so diverse taxa as *Anema* and *Peltula* spp. (Lichinomycetes), *Arctopeltis* (Lecanoromycetes, Lecanorales), and *Acarospora* spp. (Lecanoromycetes, Acarosporales) (Arup & Grube 1998, 2000, Miadlikowska et al. 2006; Reeb et al. 2004). Similarly, the subcrustose growth form is common among parmelioid lichens growing on exposed rock faces in arid and semi-arid regions and numerous species are currently accepted within the *Xanthoparmelia*-clade with such a growth form but are not closely related (Brusse 1987; Blanco et al. 2004b; Elix et al. 1986; Esslinger 1977, 2000; Hale 1989, 1990; Thell et al. 2006).

## New Combinations

Based on our phylogenetic studies and the results of the morphological and chemical analyses, we propose to add the names *Omphalodiella* and *Placoparmelia* to the list of synonyms of *Xanthoparmelia*. The following new combinations are necessary to accommodate the two species in *Xanthoparmelia*.

**Xanthoparmelia patagonica** (Henssen) Amo, Lumbsch & Crespo, *comb. nov.* Mycobank no. MB357593; *Placoparmelia patagonica* Henssen, Lichenologist 24: 134. 1992.

**Xanthoparmelia peltata** Amo, Lumbsch & Crespo, *nom. nov.* Mycobank no. MB354985; *Omphalodiella patagonica* Henssen, Lichenologist 23: 335. 1991, non *Xanthoparmelia patagonica* (Henssen) Amo, Lumbsch & Crespo, Bryologist 113: XXX.

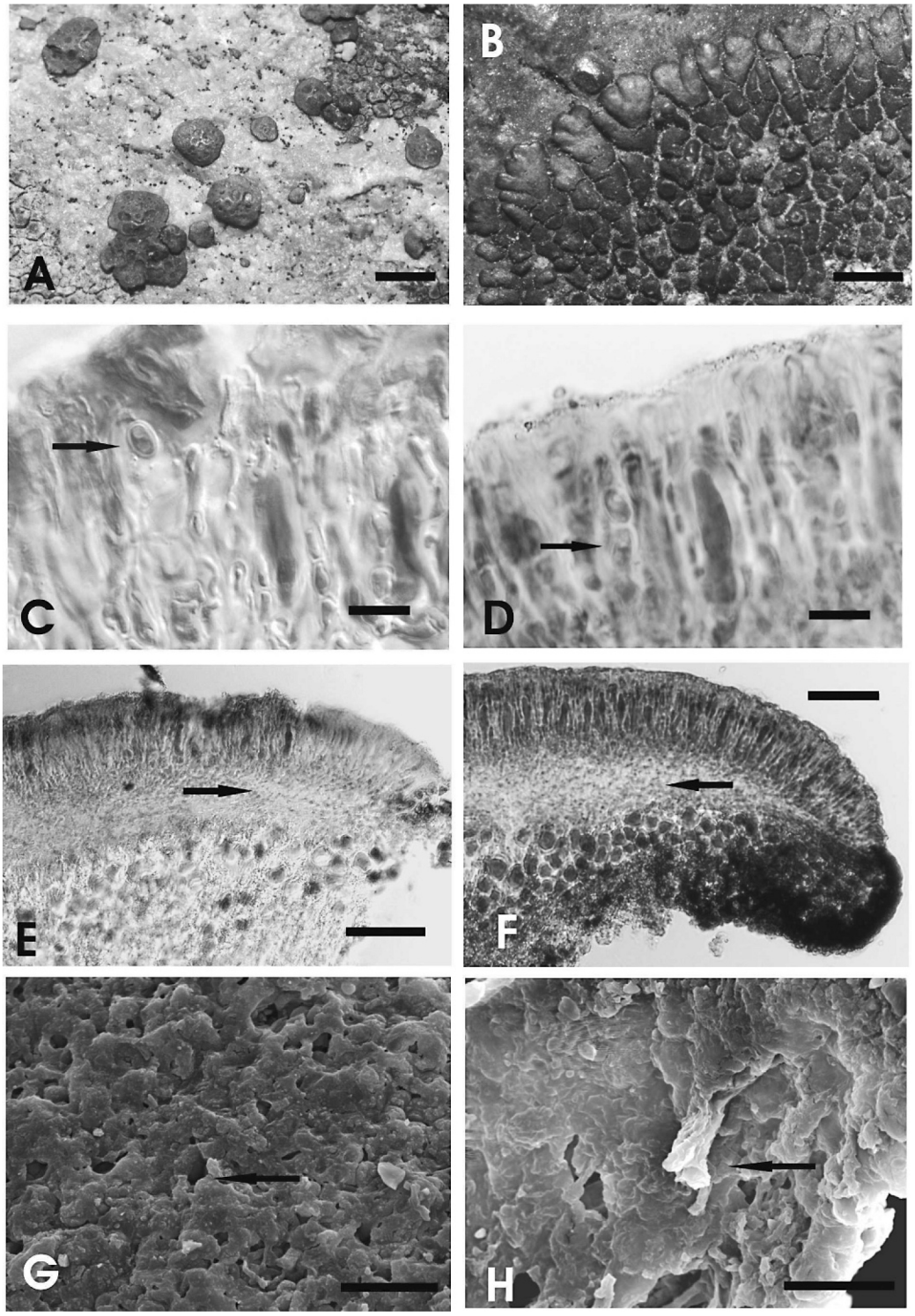


**Figure 2.** Morphology and anatomy of *Omphalodiella* and *Placoparmelia*. **A & B**. Habit, A. *Omphalodiella patagonica*. B. *Placoparmelia patagonica*. **C & D**. Ascospores (arrows showing ascospores with arachiform vacuolar body), C. *Omphalodiella patagonica*. D. *Placoparmelia patagonica*. **E & F**. Cross sections through apothecia. Arrow is marking the hyaline subhymenium. E. *Omphalodiella patagonica*. F. *Placoparmelia patagonica*. **G & H**. SEM picture of thallus surface. G. *Omphalodiella patagonica* upper surface; arrow showing pored epicortex. H. *Placoparmelia patagonica* lower surface at lobe ends with polysaccharide matrix; arrow showing small hyphal anchorage (rhizines). Scale bars: A & B = 1 mm, C & D. = 10 µm, E & F. = 50 µm, G & H. = 20 µm.

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ms received September 27, 2009; accepted March 2, 2010.

**Appendix 1.** GenBank accession numbers (nuITS, nuLSU, mitSSU) for the sequences of Parmelioid exemplars included in the phylogenetic analysis, along with the locality and voucher information of the original samples.

*Flavoparmelia caperata*, Spain, Teruel, MAF 6045, AY581059, AY578922, AF351158; *F. flaventior*, Spain, Teruel, MAF6046, AY581060, AY578923, AF351164; *Parmotrema haitiense*, Australia, ACT, MAF 7656, AY581056, AY578919, AY582296; *P. fistulatum*, Uruguay, Maldonado, MAF 7655, AY581057, AY578920, AY582297; *P. pilosum*, Uruguay, Maldonado, MAF 7656, AY581056, AY578919, AY582296; *Punctelia borreri*, Portugal; Castello Vide, MAF 9919, AY581088, AY578954, AY582324; *P. subrudecta*, Portugal, Nazaré, MAF 9918, AY581089, AY578955, AY582325; *X. angustiphylla*, USA, Illinois, MAF 6768, AY581092, AY578958, AY582328; *X. atticoides*, USA, Arizona, MAF 6744, AY581066, AY578929, AY582302; *X. azaniensis*, South Africa, Cape Region, MAF 14269, EF042900, EF042910, EF02478; *X. brachinaensis*, Australia, South Australia, Elix 30651, AY581062, AY578925, ----; *X. conspersa*, Spain, Zamora, MAF 6793, AY581096, AY578962, AF351186; *X. crespoae* (1), Australia, New South Wales, MAF 7524, AY581097, AY578963, AY582332; *X. crespoae* (2), Australia, New South Wales, MAF 7440, AY581098, AY578964, AY582333; *X. delisei* (1), Spain, Zamora, MAF 7659, AY581068, AY578931, AY582304; *X. delisei* (2), Australia, New South Wales, MAF 7432, AY581067, AY578930, AY582303; *X. digitiformis*, Australia, ;ACT, MAF 7525, AY581099, AY578965, AY582334; *X. exornata*, South Africa, Cape Region, MAF 14266, EF042908, EF108318, EF025485; *X. glabrans*, Australia, ;ACT, MAF 7665, AY581069, AY578932, AY582305; *X.* aff. *glabrans*, Spain, Zaragoza, MAF 9912, AY581072, AY578935, AY582308; *X. hottentota*, South Africa, Cape Region, MAF 14267, EF042909, EF042919, EF025486; *X. hueana*, Namibia, Swakopmund, GZU 46511, AY581090, AY578956, AY582326; *X. isidiovagans*,

Spain, Guadalajara, MAF 9956, AY581094, AY578960, AY582330; *X. lineola*, USA, Arizona, MAF 9955, ----, AY578970, AY582338; *X. lithophila*, Australia, New South Wales, MAF 6900, AY581077, AY578941, AF351171; *X. lithophiloides*, Australia, ACT, MAF 7471, AY581078, AY578942, AY582314; *X. loxodes* (1), Spain, Salamanca, MAF 7072, AY581076, AY578940, AY582313; *X. loxodes* (2), Spain, Zamora, MAF 6206, AY581070, AY578933, AY582306; *X. mougeotii* (1), Spain, Zamora, MAF 6802, AY37006, AY578966, AY582335; *X. mougeotii* (2), Spain, La Rioja, MAF 9916, AY581100, AY578967, AY582336; *X. murina*, Australia, New South Wales, MAF 9915, AY581079, AY578943, AY582315; *X. notata*, Australia, ACT, MAF 7521, AY581101, AY578968, AY582337; *X. norcapnodes*, Australia, ACT, MAF 7532, AY581080, AY578944, AY582316; *X. ovealmbornii*, South Africa, Cape Region, MAF 14268, EF042901, EF042911, EF025479; *X. pokornyi* (1), Spain, Guadalajara, MAF 6052, AY037005, AY578934, AY582307; *X. pokornyi* (2), Spain, Zaragoza, MAF 9908, AY581075, AY578939, AY582312; *X. protomatrae*, Spain, Zamora, MAF 6216, AY581104, AY578972, AY582339; *X. pulla*, Spain, Madrid, MAF 6794, AY581071, AJ 421433, AF351169; *X.* aff. *pulloides*, Spain, Zamora, MAF 6784, AY037004, AY578936, AY582309; *X. reptans*, Australia, New South Wales, MAF 7522, AY581102, AY578969, ---; *X. semiviridis*, Australia, New South Wales, MAF 6876, AY581058, AY578921, AF351158; *X. scotophylla*, Australia, South Australia, Elix 30650, AY581081, AY578945, AY582317; *X. stenophylla*, Spain, Guadalajara, MAF 9917, AY581093, AY578959, AY582329; *X. subdiffluens*, Spain, Soria, MAF 9910, AY581105, AY578973, AY582340; *X. subincerta*, Australia, ACT, MAF 7494, AY581073, AY578937, AY582310; *X. sublaevis*, Spain, Canary Islands (Tenerife), MAF 7460, AY581106, AY578974, AY582341; *X. subprolixa*, Australia, ACT, MAF 7667, AY581074, AY578938, AY582311; *X. subspodochroa*, Australia, New South Wales, MAF 7463, AY581082, AY578946, AY582318; *X. subverrucigera*, Spain, Zaragoza, MAF 9957, AY581091, AY578957, AY582327; *X. tegeta*, Australia, ACT, MAF 7523, AY581107, AY578975, AY582342; *X. tinctina* (1), Spain, Madrid, MAF 6070, AY581108, AY578976, AY582343; *X. tinctina* (2), Spain, Barcelona, BCN-13861, AY581109, AY578977, AY582344; *X. tinctina* (3), Spain, Barcelona, BCN-13862, AY581110, AY578978, AY582345; *X. transvaalensis*, Spain, Zaragoza, MAF 9841, AY581095, AY578961, AY582331; *X. verrucigera*, Spain, Gerona, MAF 9920, AY581111, AY578979, AY582346; *X. vicentei* (1), Spain, Salamanca, MAF 7248, AY581112, AY578980, AY582347; *X. vicentei* (2), Spain, Salamanca, MAF 9954, AY581103, AY578971, ----.

# El género *Karoowia* (Parmeliaceae, Ascomycota) incluye clados no relacionados entre si agrupados dentro de *Xanthoparmelia*

Guillermo Amo de Paz, H. Thorsten Lumbsch, Paloma Cubas, John A. Elix & Ana Crespo

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## Resumen

La morfología del talo ha jugado tradicionalmente un papel importante en la clasificación de los hongos liquenizados. Es frecuente que en la actual clasificación de los líquenes cada género exhiba un único biotipo. Sin embargo, los datos moleculares están mostrando que los diferentes linajes evolutivos poseen variabilidad en cuanto a la morfología del talo. Usando una combinación de secuencias de nuITS, nuLSU y mtSSU de ADN ribosomal hemos evaluado las relaciones filogenéticas entre el género subcrustáceo *Karoowia* y el género *Xanthoparmelia,* que es principalmente foliáceo. El análisis filogenético usando parsimonia, máxima verosimilitud y estadística bayesiana muestra que las especies de *Karoowia* no forman un grupo monofilético sino que se agrupan en diferentes clados dentro de *Xanthoparmelia*. La monofilia del género *Karoowia*, bien como un clado independiente o anidado dentro del clado 'xanthoparmelia', se ha rechazado mediante un test de hipótesis. Estos resultados sugieren que los caracteres fenotípicos usados para describir *Karoowia* han sido sobreestimados, ya que el talo subcrustáceo ha evolucionado independientemente en varios linajes del clado 'xanthoparmelia'. Otros caracteres usados para describir *Karoowia*, como la presencia de conidios cilíndricos, no son exclusivos de este género sino que también aparecen en *Xanthoparmelia*. Del mismo modo, especies de *Karoowia* muestran conidios bifusiformes. Las diferencias en la morfología de la ricinas entre ambos géneros son mínimas y cuantitativas. Además las especies de *Karoowia* presentan el polisacárido de pared tipo 'Xanthoparmelia lichenana' y un cuerpo vacuolar araquiforme en las ascósporas, que son caracteres diagnósticos del género *Xanthoparmelia*. Consecuentemente, proponemos la sinonimia de *Karoowia* en *Xanthoparmelia*. Se han propuesto 15 nuevas combinaciones en *Xanthoparmelia* y un nuevo nombre, *Xanthoparmelia mucinae* para la *Karoowia squamatica*.

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# The genus *Karoowia* (Parmeliaceae, Ascomycota) includes unrelated clades nested within *Xanthoparmelia*

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**Abstract.** Thallus morphology has traditionally played a major role in the classification of lichenised fungi. We have used a combined dataset of nuITS, nuLSU and mtSSU rDNA sequences to evaluate the phylogenetic relationships between the subcrustose genus *Karoowia* and the mostly foliose genus *Xanthoparmelia.* Our phylogenetic analyses using maximum parsimony, maximum likelihood and a Bayesian approach show that *Karoowia* species do not form a monophyletic group but cluster in different clades nested within *Xanthoparmelia.* The monophyly of *Karoowia* either as a separate clade from *Xanthoparmelia*, or nested within *Xanthoparmelia* is significantly rejected using alternative hypothesis testing. These results suggest that the usefulness of the phenotypic features used to define *Karoowia* has been overestimated because the subcrustose growth form has evolved independently in several clades within *Xanthoparmelia.* Other characters used to circumscribe *Karoowia*, such as the presence of cylindrical conidia, also occur in *Xanthoparmelia*, and the differences in rhizine morphology are minimal. Consequently, we propose to reduce *Karoowia* to synonymy with *Xanthoparmelia.* The enlarged genus is characterised by the presence of *Xanthoparmelia*-type lichenan in the hyphal cell walls and the presence of an arachiform vacuolar body in the ascospores. Fifteen new combinations in *Xanthoparmelia* and the new name *Xanthoparmelia mucinae* for *Karoowia squamatica* are made.

## Introduction

Lichen-forming fungi are distinguished by their extensive vegetative thalli to host the photosynthetic symbiotic partner, unlike non-lichenised fungi that often form simple mycelia. Thus, it is not surprising that lichen taxonomists have used vegetative characters for the classification of lichenised fungi from the beginning. Even in the current classification most of the accepted genera in lichenised fungi exhibit a single growth form. However, molecular data have assisted in identifying clades that show variability within these characters. Examples in which species with different growth forms were shown to belong to a single genus (and in *Sticta*, even the same fungal species) include *Roccellina* (Tehler and Irestedt 2007), *Stereocaulon* (Högnabba 2006), *Sticta* (James and Henssen 1976; Armaleo and Clerc 1991), and *Xanthoparmelia* (Blanco *et al*. 2004*b*). The latter genus belongs to the family Parmeliaceae. This family represents one of the largest families of lichenised fungi (Hale and DePriest 1999; Crespo *et al*. 2007). In the main clade (parmelioid lichens) of the family, the circumscription of genera has been disputed, with some authors only recognising a single genus (*Parmelia s. lat*.) (Poelt and Vězda 1981; Clauzade and Roux 1985; Eriksson and Hawksworth 1986; Llimona and Hladun 2001). Other lichenologists have suggested classifying the ~1500 described species in the clade into more than 35 genera (Hale 1984; Elix 1993; Hale and DePriest 1999). This is mainly due to the absence of obvious ascomatal characters and the wide use of vegetative morphological and chemical characters in the classification of parmelioid lichens. Recent molecular studies have helped to identify monophyletic clades in parmelioid lichens and to re-evaluate phenotypic characters previously used to circumscribe genera (Crespo and Cubero 1998; Blanco *et al*. 2004*a*, 2004*b*, 2005, 2006; Thell *et al*. 2004, 2006; Divakar *et al*. 2006, 2010; Crespo *et al*. 2007, 2010; Del Prado *et al*. 2007; Amo de Paz *et al*. 2010). One of the major clades identified among parmelioid lichens is the *Xanthoparmelia*-clade (Blanco *et al*. 2006; Lumbsch *et al*. 2008). This clade includes more than 800 species occurring on siliceous rocks or soil mainly in arid to semiarid regions, with a centre of diversity in the southern hemisphere. The clade is characterised by having cell wall polysaccharides of the *Xanthoparmelia*-type, small ascospores with an arachiform vacuolar body (Del Prado *et al*. 2007), and the presence of a pored epicortex (Blanco *et al*. 2004*b*, 2006).

Within this clade, currently two genera are accepted, viz. *Karoowia* Hale and *Xanthoparmelia* (Vain.) Hale. The latter genus has recently been expanded following morphological and molecular studies that found previously segregated genera

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nested within *Xanthoparmelia*. These genera were subsequently reduced to synonymy with *Xanthoparmelia.* This includes genera previously recognised based on cortical chemistry such as *Neofuscelia* Essl. and *Paraparmelia* Elix & J. Johnst., growth form such as *Almbornia* Essl., *Chondropsis* Nyl. ex Cromb., *Placoparmelia* Hessen, *Omphalodiella* Hessen and *Xanthomaculina* Hale, and peculiar features of the upper surface such as *Namakwa* Hale (Hawksworth and Crespo 2002; Elix 2003; Blanco *et al*. 2004*b*; Thell *et al*. 2006; Amo de Paz *et al*. 2010). Species of *Xanthoparmelia* form a large clade which is well supported as being monophyletic. The branches of phylogenetic trees within this clade are comparably shorter than in other parmelioid groups. These facts have been interpreted as resulting from a rapid adaptive radiation after a shift towards drier habitats at the base of the *Xanthoparmelia* clade (Lumbsch *et al*. 2008). This habitat shift presumably led to different morphological and/or chemical adaptations that have been misinterpreted in previous classifications.

The genus *Karoowia* currently includes 16 species (Elix 1997, 1999, 2000). It was described (Hale 1989) to accommodate 19 subcrustose species similar to *Xanthoparmelia* but which differed in having rhizoid-like structures instead of true rhizines and producing longer cylindrical conidia (6–12 µm long) in comparison to the shorter bifusiform to bacilliform (4–9 µm long) conidia present in *Xanthoparmelia*. However, previous molecular studies showed that two *Karoowia* species were nested within *Xanthoparmelia* (Blanco *et al*. 2004*b*; Thell *et al*. 2004). We have now extended the taxon sampling to address the phylogenetic position of *Karoowia* using a combined dataset of nuclear Internal Transcribed Spacer (nuITS), nuclear Large SubUnit (nuLSU) and mitochondrial Small SubUnit (mtSSU) of the rDNA sequences. Further, we reexamined the morphology and chemistry of *Karoowia* species to evaluate characters that have previously been used to segregate *Karoowia* and *Xanthoparmelia.*

## Materials and methods

### *Chemical analyses*

The chemical constituents were identified using thin-layer chromatography (TLC) (Culberson 1972; Culberson *et al*. 1981; Culberson and Johnson 1982), and gradient-elution high performance liquid chromatography (HPLC) (Feige *et al*. 1993).

### *Molecular studies*

Eighty-four specimens of *Karoowia* (eight species) and *Xanthoparmelia* (fifty-nine species) were used for the molecular study. The delimitation of species in the group follows Hale (1989). We included several specimens of the same species to assess intraspecific variability.

We used sequences of the nuITS rDNA, nuLSU rDNA and mtSSU rDNA of forty-eight specimens of *Xanthoparmelia* and two specimens of *Karoowia* from previous studies (Blanco *et al*. 2004*b*, 2006; Crespo *et al*. 2007). We generated 34 sequences of nuITS rDNA, 33 of nuLSU rDNA and 31 mtSSU rDNA from 34 *Karoowia* specimens and other *Xanthoparmelia* species. The GenBank accession numbers, localities and voucher numbers are shown in Table 1. Sequences of seven species of parmelioid lichens outside the *Xanthoparmelia*-clade were downloaded from GenBank and used as the outgroup for the analyses. The outgroup selection was based on previous phylogenetic studies (Crespo *et al*. 2007; Lumbsch *et al*. 2008).

Frozen lobes of the specimens were crushed with sterile glass pestles. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions and modifications as detailed elsewhere (Crespo *et al*. 2001). The following primers were used: for nuITS rDNA: ITS 1F (Gardes and Bruns 1993), ITS1-LM (Myllys *et al*. 1999), ITS4 (White *et al*. 1990) and ITS2-KL (Lohtander *et al*. 1998); for nuLSU rDNA: LR0R and LR5 (Vilgalys and Hester 1990); and for mtSSU: mrSSU1 and mrSSU3R (Zoller *et al*. 1999).

For each amplification we used a reaction mixture of 50 µL, containing 5 µL of 10× DNA polymerase buffer (Biotools, Madrid, Spain) (2 mM $MgCl_2$, 75 mM TRIS-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 µL of each primer (10 µM), 1.25 µL of DNA polymerase (1U µL$^{-1}$), 1 µL of dNTPs containing 10 mM of each base (Biotools), 10 µL of DNA (third elution of DNA extraction) and 27.75 µL $dH_2O$. Amplifications were carried out in an automatic thermocycler (Techne Progene 3000, Cambridge, UK). Programs with the following steps were performed: Step 1: initial denaturation at 94°C for 5 min; Step 2: 35 cycles of 94°C for 1 min, 60°C (nuITS rDNA with ITS1-LM and ITS-2KL; and mtSSU rDNA), 56°C (nuITS rDNA with ITS1F and ITS4) or 58°C (nuLSU rDNA) for 1 min, and 72°C for 1.5 min; Step 3: final extension at 72°C for 5 min. We used a DNA Purification Kit (Favorgen, Pintung, Taiwan) to clean the PCR products. The cleaned PCR products were sequenced with the same primers as in the PCR reactions, using the ABI Prism Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA) with the following program: initial denaturation at 94°C for 3 min and 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems). Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually adjusted.

### *Sequence alignments and phylogenetic analyses*

The sequences were aligned separately for each dataset using Muscle 3.6 (Edgar 2004) and ambiguously aligned positions were removed manually. Gaps were treated as missing data.

We performed phylogenetic analyses using maximum likelihood (ML), maximum parsimony (MP) and a Bayesian approach. To test for potential conflicts, parsimony bootstrap analyses were performed on each individual dataset, and bootstrap trees with a 70% threshold were examined for conflicts (Lutzoni *et al*. 2004).

ML analyses were performed using Garli 0.96 (Zwickl 2006) using the GTR+I+G model. MP analyses were performed using the program PAUP* 4.0b (Swofford 2002) with 200 random additions and characters unordered and equally weighted. MulTrees option and branch swapping using TBR were in effect. Bootstrap analyses (Felsenstein 1985) were performed with 2000 pseudoreplicates with the same settings as the MP search. To assess homoplasy levels, consistency index (*CI*), retention index (*RI*) and rescaled consistency index (*RC*) were calculated from each parsimony search.

**Table 1. GenBank accession numbers, locality and voucher information of the included samples**
Species with new sequences are shown in bold

| Species | Locality | Herbarium acc. no. | GenBank acc. no. | | |
|---|---|---|---|---|---|
| | | | nuITS | nuLSU | mtSSU |
| *Flavoparmelia caperata* | Spain, Teruel | MAF 6045 | AY581059 | AY578922 | AF351158 |
| *Flavopunctelia flaventior* | Spain, Teruel | MAF 6046 | AY581060 | AY578923 | AF351164 |
| ***Karoowia adhaerens* 1** | South Africa, Western Cape | MAF 16212 | HM125744 | HM125766 | HM125722 |
| ***K. adhaerens* 2** | South Africa, Western Cape | MAF 16213 | HM125746 | HM125768 | HM125724 |
| ***K. perspersa* 1** | South Africa, Western Cape | MAF 15543 | GU903338 | GU903347 | GU903328 |
| ***K. perspersa* 2** | South Africa, Northern Cape | MAF 16222–1 | GU992330 | HM125772 | HM125728 |
| ***K. perspersa* 3** | South Africa, Western Cape | MAF 15538 | GU903339 | GU903348 | GU903329 |
| ***K. perspersa* 4** | South Africa, Northern Cape | MAF 15541 | GU992333 | HM125778 | HM125734 |
| ***K. perspersa* 5** | South Africa, Northern Cape | MAF 15542 | GU992329 | HM125769 | HM125725 |
| ***K. perspersa* 6** | South Africa, Northern Cape | MAF 16448 | HM125758 | HM125783 | HM125737 |
| ***K. perspersa* 7** | South Africa, Northern Cape | MAF 15528 | GU992328 | HM125762 | HM125718 |
| ***K. perspersa* 8** | South Africa, Western Cape | MAF 15539 | GU992332 | HM125776 | HM125732 |
| ***K. ralla*** | South Africa, Northern Cape | MAF 15537 | GU903337 | GU903346 | GU903327 |
| *K. saxeti* 1 | Taiwan | APROOT 53350 | AY581063 | AY578926 | AY582299 |
| ***K. saxeti* 2** | India, Karnatka | MAF 16224 | HM125739 | HM125760 | HM125716 |
| ***K. scitula* 1** | South Africa, Northern Cape | MAF 15536 | GU903333 | GU903342 | GU903325 |
| ***K. scitula* 2** | South Africa, Western Cape | MAF 15535–1 | HM125741 | HM125763 | HM125719 |
| ***K. scitula* 3** | South Africa, Northern Cape | MAF 15535–2 | GU903334 | GU903343 | GU903324 |
| ***K. subchalybaeizans* 1** | South Africa, Northern Cape | MAF 15530–1 | GU903336 | GU993345 | GU903323 |
| ***K. subchalybaeizans* 2** | South Africa, Western Cape | MAF 15532 | HM125750 | HM125774 | HM125730 |
| ***K. subchalybaeizans* 3** | South Africa, Western Cape | MAF 15533–1 | HM125743 | HM125765 | HM125721 |
| ***K. subchalybaeizans* 4** | South Africa, Western Cape | MAF 15533–2 | GU903331 | GU903340 | GU903322 |
| ***K. subchalybaeizans* 5** | South Africa, Western Cape | MAF 16443 | HM125759 | HM125784 | HM125738 |
| ***K. subchalybaeizans* 6** | South Africa, Western Cape | MAF 15534 | HM125747 | HM125770 | HM125726 |
| ***K. subchalybaeizans* 7** | South Africa, Northern Cape | MAF 15531–1 | HM125745 | HM125767 | HM125723 |
| ***K. subchalybaeizans* 8** | South Africa, Northern Cape | MAF 15531–2 | HM125748 | HM125771 | HM125727 |
| ***K.* aff. *supposita* 1** | South Africa, Western Cape | MAF 16206–1 | HM125749 | HM125773 | HM125729 |
| ***K.* aff. *supposita* 2** | South Africa, Western Cape | MAF 16206–2 | HM125751 | HM125775 | HM125731 |
| *Parmotrema haitiense* | Australia, Australian Capital Territory | MAF 7656 | AY581056 | AY578919 | AY582296 |
| *P. fistulatum* | Uruguay, Maldonado | MAF 7655 | AY581057 | AY578920 | AY582297 |
| *P. pilosum* | Uruguay, Maldonado | MAF 7656 | AY581056 | AY578919 | AY582296 |
| *Punctelia borreri* | Portugal, Castello Vide | MAF 9919 | AY581088 | AY578954 | AY582324 |
| *P. subrudecta* | Portugal, Nazaré | MAF 9918 | AY581089 | AY578955 | AY582325 |
| *Xanthoparmelia angustiphylla* | USA, Illinois | MAF 6768 | AY581092 | AY578958 | AY582328 |
| *X. atticoides* | USA, Arizona | MAF 6744 | AY581066 | AY578929 | AY582302 |
| *X. azaniensis* | South Africa, Cape Region | MAF 14269 | EF042900 | EF042910 | EF02478 |
| *X. brachinaensis* | Australia, South Australia | Elix 30651 | AY581062 | AY578925 | – |
| *X.* aff. *caparidensis* | South Africa, Western Cape | MAF 16113 | GU461305 | GU461303 | GU461302 |
| ***X. chalybaeizans*** | South Africa, Northern Cape | MAF 15530–2 | GU903332 | GU903341 | GU903330 |
| ***X. condyloides*** | South Africa, Northern Cape | MAF 16444 | HM125754 | – | – |
| *X. conspersa* | Spain, Zamora | MAF 6793 | AY581096 | AY578962 | AF351186 |
| *X. crespoae* 1 | Australia, New South Wales | MAF 7524 | AY581097 | AY578963 | AY582332 |
| *X. delisei* 1 | Spain, Zamora | MAF 7659 | AY581068 | AY578931 | AY582304 |
| *X. delisei* 2 | Australia, New South Wales | MAF 7432 | AY581067 | AY578930 | AY582303 |
| *X. digitiformis* | Australia, Australian Capital Territory | MAF 7525 | AY581099 | AY578965 | AY582334 |
| ***X.* aff. *epacridea*** | South Africa, Western Cape | MAF 16208 | HM125752 | HM125777 | HM125733 |
| *X. exornata* | South Africa, Northern Cape | MAF 14266 | EF042908 | EF108318 | EF025485 |
| *X. glabrans* | Australia, Australian Capital Territory | MAF 7665 | AY581069 | AY578932 | AY582305 |
| *X.* aff. *glabrans* | Spain, Zaragoza | MAF 9912 | AY581072 | AY578935 | AY582308 |
| ***X. greytonensis*** | South Africa, Western Cape | MAF 16210 | HM125755 | HM125780 | – |
| *X. hottentota* | South Africa, Western Cape | MAF 14267 | EF042909 | EF042919 | EF025486 |
| *X. hueana* | Namibia, Swakopmund | GZU 46511 | AY581090 | AY578956 | AY582326 |
| *X. isidiovagans* | Spain, Guadalajara | MAF 9956 | AY581094 | AY578960 | AY582330 |
| *X. lineola* | USA, Arizona | MAF 9955 | – | AY578970 | AY582338 |
| *X. lithophila* | Australia, New South Wales | MAF 6900 | AY581077 | AY578941 | AF351171 |
| *X. lithophiloides* | Australia, Australian Capital Territory | MAF 7471 | AY581078 | AY578942 | AY582314 |
| *X. loxodes* 1 | Spain, Salamanca | MAF 7072 | AY581076 | AY578940 | AY582313 |
| *X. loxodes* 2 | Spain, Zamora | MAF 6206 | AY581070 | AY578933 | AY582306 |

**Table 1.** (*continued*)

| Species | Locality | Herbarium acc. no. | GenBank acc. no. | | |
|---|---|---|---|---|---|
| | | | nuITS | nuLSU | mtSSU |
| *X. mougeotii* 1 | Spain, Zamora | MAF 6802 | AY37006 | AY578966 | AY582335 |
| *X. mougeotii* 2 | Spain, La Rioja | MAF 9916 | AY581100 | AY578967 | AY582336 |
| *X. murina* | Australia, New South Wales | MAF 9915 | AY581079 | AY578943 | AY582315 |
| ***X. neotumidosa*** | South Africa, Western Cape | MAF 16442 | HM125758 | HM125782 | HM125736 |
| *X. notata* | Australia, Australian Capital Territory | MAF 7521 | AY581101 | AY578968 | AY582337 |
| *X. norcapnodes* | Australia, Australian Capital Territory | MAF 7532 | AY581080 | AY578944 | AY582316 |
| *X. ovealmbornii* | South Africa, Cape Region | MAF 14268 | EF042901 | EF042911 | EF025479 |
| *X. peltata* | Argentina, Río Negro | Lumbsch 11036a | DQ980021 | DQ923670 | DQ923643 |
| *X. pokornyi* 1 | Spain, Guadalajara | MAF 6052 | AY037005 | AY578934 | AY582307 |
| *X. pokornyi* 2 | Spain, Zaragoza | MAF 9908 | AY581075 | AY578939 | AY582312 |
| *X. protomatrae* | Spain, Zamora | MAF 6216 | AY581104 | AY578972 | AY582339 |
| *X. pulla* | Spain, Madrid | MAF 6794 | AY581071 | AJ 421433 | AF351169 |
| *X.* aff. *pulloides* | Spain, Zamora | MAF 6784 | AY037004 | AY578936 | AY582309 |
| *X. reptans* | Australia, New South Wales | MAF 7522 | AY581102 | AY578969 | – |
| *X. semiviridis* | Australia, New South Wales | MAF 6876 | AY581058 | AY578921 | AF351158 |
| *X. scotophylla* | Australia, South Australia | Elix 30650 | AY581081 | AY578945 | AY582317 |
| *X. stenophylla* | Spain, Guadalajara | MAF 9917 | AY581093 | AY578959 | AY582329 |
| ***X. subamplexuloides*** | Kenia, Rift Valley Province | MAF 16441 | HM125753 | HM125779 | HM125735 |
| *X. subdiffluens* | Spain, Soria | MAF 9910 | AY581105 | AY578973 | AY582340 |
| *X. subincerta* | Australia, Australian Capital Territory | MAF 7494 | AY581073 | AY578937 | AY582310 |
| *X. sublaevis* | Spain, Canary Islands (Tenerife) | MAF 7460 | AY581106 | AY578974 | AY582341 |
| *X. subprolixa* | Australia, Australian Capital Territory | MAF 7667 | AY581074 | AY578938 | AY582311 |
| *X. subpodochroa* | Australia, New South Wales | MAF 7463 | AY581082 | AY578946 | AY582318 |
| *X. subverrucigera* | Spain, Zaragoza | MAF 9957 | AY581091 | AY578957 | AY582327 |
| *X. tegeta* | Australia, Australian Capital Territory | MAF 7523 | AY581107 | AY578975 | AY582342 |
| ***X. tentaculina*** | South Africa, Northern Cape | MAF 16445 | HM125756 | HM125781 | – |
| *X. tinctina* 1 | Spain, Madrid | MAF 6070 | AY581108 | AY578976 | AY582343 |
| *X. tinctina* 2 | Spain, Barcelona | BCN-13861 | AY581109 | AY578977 | AY582344 |
| ***X. tortula*** | South Africa, Northern Cape | MAF 16446 | HM125742 | HM125764 | HM125720 |
| *X. transvaalensis* | Spain, Zaragoza | MAF 9841 | AY581095 | AY578961 | AY582331 |
| *X. verrucigera* | Spain, Gerona | MAF 9920 | AY581111 | AY578979 | AY582346 |
| *X. vicentei* | Spain, Salamanca | MAF 7248 | AY581112 | AY578980 | AY582347 |
| ***X. xanthomelaena*** | Australia, New South Wales | MAF 16447 | HM125740 | HM125761 | HM125717 |

A Bayesian analysis was performed using MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001) using the GTR+I+G model. The dataset was partitioned into three parts: nuITS, nuLSU and mtSSU. Each partition was allowed to have its own parameter values (Nylander *et al*. 2004). No molecular clock was assumed. We used two simultaneous runs and four chains per run. We used the default setting of the temp parameter of the heated chains (temp = 0.2). Posterior probabilities were approximated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. The number of generations was 5 million, with every 200th tree sampled. The first 3000 generations were discarded as burn in. We used AWTY (Nylander *et al*. 2007) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. Of the remaining 44 000 trees (22 000 from each of the parallel runs) a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades with bootstrap support equal or above 70% under MP and posterior probabilities $\geq$0.95 in the Bayesian analysis were considered to be strongly supported. Phylogenetic trees were visualised using the program Treeview (Page 1996).

We employed alternative hypothesis testing to evaluate whether our dataset allowed rejection of the two null hypotheses: (1) *Karoowia* forms a monophyletic lineage, and (2) *Karoowia adhaerens* (type species) belongs to the *K. saxeti* clade. ML trees inferred from unconstrained and constrained searches were obtained using Garli 0.98 (Zwickl 2006). For the hypothesis testing, we used two different methods: (1) Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 2001), and (2) expected likelihood weight (ELW) test (Strimmer and Rambaut 2002). The SH and ELW tests were performed using Tree-PUZZLE 5.2 (Schmidt *et al*. 2002) with the combined dataset, comparing the best tree agreeing with the null hypotheses, and the unconstrained ML tree.

### *Scanning-electron microscopy (SEM)*

Lichen thalli were washed in tap water and then dehydrated (Samson *et al*. 1979). Critical point dried specimens were mounted on stubs and sputter-coated with gold. The specimens were examined using a Jeol JSM 6400 SEM.

### *Cell-wall polysaccharide analysis*

The type of lichenan in the hyphal cell walls was determined using histochemical methods (Nash *et al*. 1990; Common 1991). Thallus cross-sections were clarified using Chlorox (Neutrex, Barcelona, Spain) after wetting with a liquid detergent solution.

Subsequently they were transferred through a series of iodine solutions (20, 1.5 and 0.15% IKI) and Melzer's solution. *Xanthoparmelia*-type lichenan forms a red-brown complex with Melzer's solution, whereas isolichenan or *Parmotrema*-type lichenan forms a pale complex. With iodine solutions, *Xanthoparmelia*-type lichenan forms a dark blue complex in 0.15% IKI, whereas isolichenan forms a pale complex and *Parmotrema*-type lichenan a pale blue complex. Observations were made under a Leica S6E compound microscope.

### Additional material studied

The following material was used to study morphological and chemical characters: *Karoowia adhaerens* South Africa, D. Triebel & G. Rambold 7976 (M). *K. adligans* South Africa, 1990, D. Triebel & G. Rambold (CANB 753187). *K. brachinaensis* Australia, 2003, J.A. Elix (CANB 753188). *K. perspersa* South Africa, 1986, T.H. Nash (CANB 753194.1); 1986, F. Brusse (BM). *K. ralla* South Africa, isotype (CANB 9003751.2); South Africa, 1986, M.E. Hale (CANB 753192.1). *K. saxeti* South Africa, 1990, D. Triebel & G. Rambold 7390 (M). *K. scitula* South Africa, 1990, F. Brusse (BM, CANB 753196). *Parmelia supposita* South Africa, isotype, F. Brusse (BM). *Xanthoparmelia adhaerens* South Africa, isotype, A.E. Eaton (BM).

## Results

### Molecular studies

Ninety-eight new sequences were obtained for this study (Table 1). The MP bootstrap analyses of the single datasets did not identify any conflicts (i.e. well supported differences in the topology) among the analyses; hence a multi-gene dataset was analysed. A matrix of 1896 unambiguously aligned nucleotide characters, with 510 positions in the nuITS, 849 positions in the nuLSU and 537 positions in the mtSSU, was used for the analyses. The number of constant characters was 1485, and 276 characters were parsimony-informative. The MP analysis of the combined dataset yielded 43 212 most parsimonious trees, 1289 steps long ($CI = 0.424$, $RI = 0.726$, $RC = 0.308$). The ML tree had a likelihood value of $\ln L = -9892.5210$.

In the B/MCMC analysis of the combined dataset the likelihood parameters had the following values averaged for the three partitions (±standard deviation): base frequencies $\pi(A) = 0.266$ (±0.0001), $\pi(C) = 0.224$ (±0.0002), $\pi(G) = 0.258$ (±0.0001), $\pi(T) = 0.254$ (±0.0001), $\ln L = -10177.132$ (1.0265), and the gamma shape parameter $\alpha = 0.546$ (±0.0002). The topology of the trees from the ML, MP (strict consensus tree) and Bayesian analyses did not show any conflict and hence only the ML tree is shown here (Fig. 1), with MP bootstrap values ≥70% and posterior probabilities ≥0.95 added.

In the phylogenetic tree (Fig. 1) the *Xanthoparmelia*-clade is well supported. *Karoowia* is polyphyletic and the species occur in six different clades nested within the *Xanthoparmelia*-clade. Table 2 shows the likelihood of alternative hypotheses: (1) monophyletic *Karoowia* nested within *Xanthoparmelia*, and (2) the type species *Karoowia adhaerens* joins with *K. saxeti* outside the clade containing *Xanthoparmelia* and the rest of *Karoowia*. Both hypotheses are significantly rejected by our dataset. Some species, such as *Karoowia perspersa* and *K. subchalybaeizans*, show remarkable genetic diversity.

### Morphological and chemical studies

The rhizines in *Karoowia* are smaller and less developed than in *Xanthoparmelia* although the fine structure under SEM is similar in both groups (Fig. 2). Similarly, the ascospores in the *Karoowia* species examined show an arachiform vacuolar body (Fig. 3), a character typical of *Xanthoparmelia* spores.

*Karoowia* was principally characterised by the combination of a subcrustose thallus and cylindrical conidia (Hale 1989), whereas *Xanthoparmelia* was considered to have bifusiform to bacilliform conidia and a foliose thallus. However, in our literature survey (Table 3) we do not confirm such a correlation and found a more complex pattern of conidial variability (Fig. 4). The combination of characters for the two genera as used by Hale (1989) when describing *Karoowia* is present in the majority of *Karoowia* (84%) and *Xanthoparmelia* (66%) species. However, several *Xanthoparmelia* species (17%) have a subcrustose thallus and the conidial morphology is variable (cylindrical to bifusiform). The mapping of growth forms and conidial morphology on the phylogenetic tree (Fig. 1) shows that these characters are homoplasious within the *Xanthoparmelia*-clade.

The results of the histochemical test for cell wall polysaccharides are summarised in Table 4. All species of *Karoowia* and *Xanthoparmelia* examined show reactions typical for the *Xanthoparmelia*-type lichenan.

## Discussion

Our phylogenetic analyses show that *Karoowia* species do not form a monophyletic group but cluster with different clades within *Xanthoparmelia.* This clearly indicates that the subcrustose thallus has evolved several times in different clades of *Xanthoparmelia.* These results confirm previous findings that growth form is a poor phylogenetic discriminator at the generic level in the *Xanthoparmelia*-clade. Genera described based on growth form that are currently synonymised with *Xanthoparmelia* include the subfruticose *Almbornia*, umbilicate *Xanthomaculina*, and peltate *Omphalodiella* (Thell *et al*. 2006; Amo de Paz *et al*. 2010).

The correlation of subcrustose growth form and conidial morphology that was used to support the distinctiveness of *Karoowia* (Hale 1989) was not supported in our studies. The *Xanthoparmelia mougeotii* clade illustrates this point (arrow head in Fig. 1). This clade includes the type species of *Karoowia* (*K. adhaerens*). It shows remarkable variability in conidial morphology and growth forms. For example, the subcrustose *K. adhaerens* and the foliose *X. tegeta* have cylindrical conidia whereas the closely related subcrustose *X. mougeotii* has bifusiform conidia.

Morphological and chemical characters corroborate the results of the phylogenetic analyses based on DNA sequence data placing *Karoowia* within *Xanthoparmelia*. Characters that were used to support a distinction between *Karoowia* and *Xanthoparmelia*, such as the morphology of the rhizines and conidia, are shown to be variable but not different. In the case of the conidia, shape and size is more variable than previously thought. Characters that have been shown to be more reflective

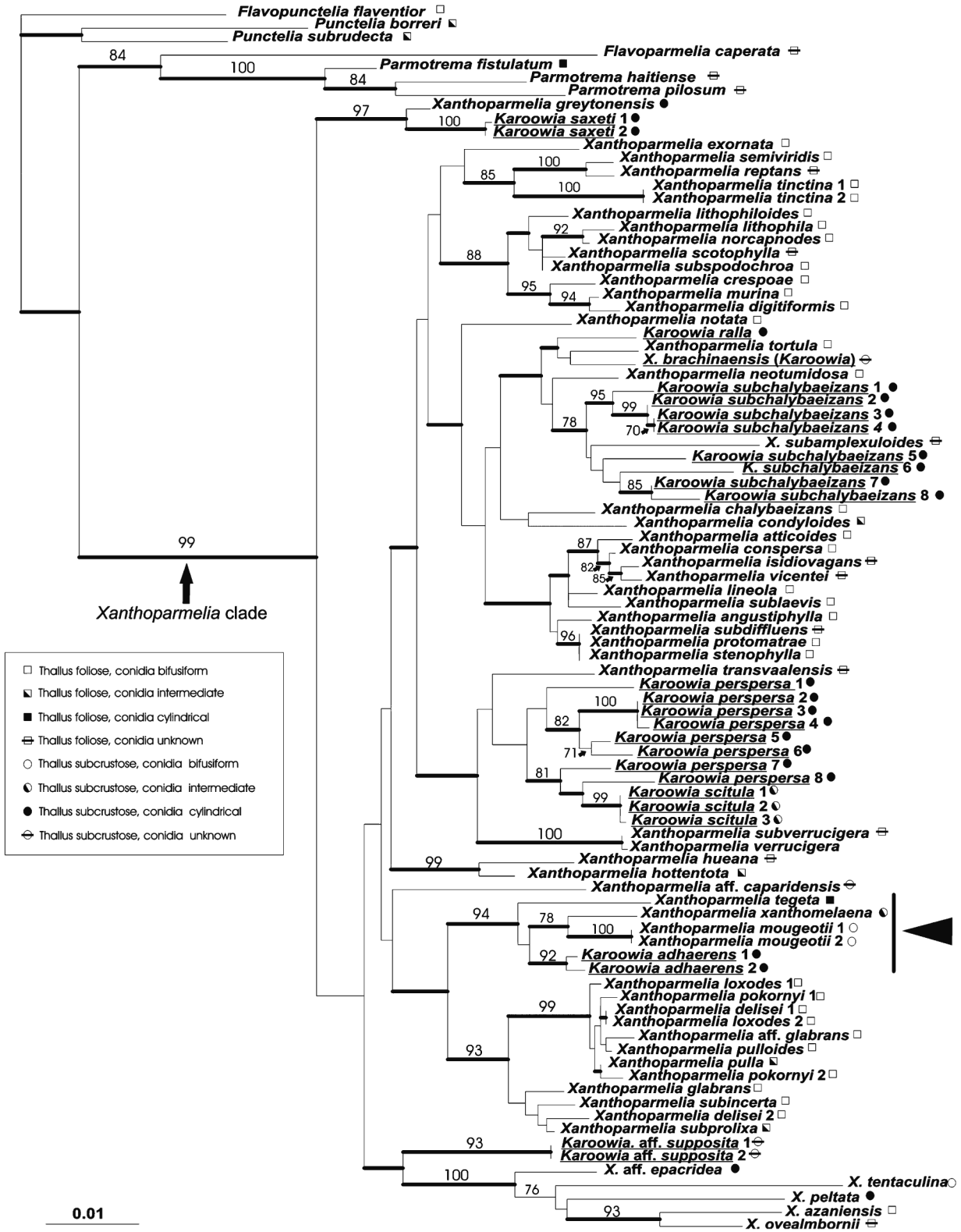


**Fig. 1.** Phylogenetic tree showing the placement of *Karoowia* and *Xanthoparmelia* species. This is a maximum-likelihood (ML) tree inferred from a combined dataset of nuITS, nuLSU, and mtSSU rDNA sequences. Bootstrap values equal to or greater than 70% under MP are indicated above branches. Branches with posterior probabilities equal to or above 0.95 are in bold. Growth form and conidia type of each species are represented. Arrow head indicates the clade of the type species *Karoowia adhaerens*.

**Table 2. Results of the alternative-hypothesis testing**
* probabilities significant at <0.05 and, ** <0.001. SH = Shimodaira–Hasegawa test (2001), ELW = expected likelihood weights test (Strimmer and Rambaut 2002)

| Null hypothesis | Probability | |
|---|---|---|
| | p-SH | c-ELW |
| *Karoowia* forming a monophyletic lineage | 0.0001** | 0.0001** |
| *Karoowia adhaerens* belongs to the *K. saxeti* clade | 0.002* | 0.0001** |

of phylogeny in Parmeliaceae, such as cell wall polysaccharides and ascospore-types (Common 1991; Common and Brodo 1995; Blanco *et al*. 2006; Crespo *et al*. 2007; Del Prado *et al*. 2007), are identical in *Karoowia* and *Xanthoparmelia*.

This study provides further evidence that the growth form of lichens exhibits a high degree of plasticity at the generic level (Arup and Grube 1998, 2000; Grube and Arup 2001; Gaya *et al*. 2003; Søchting and Lutzoni 2003; Blanco *et al*. 2004*b*; Högnabba 2006; Tehler and Irestedt 2007; Amo de Paz *et al*. 2010; Parnmen *et al*. 2010). The morphological divergence in thalline morphology is most probably caused by adaptations to particular ecological factors (Grube and Hawksworth 2007); ecophysiological studies comparing different growth forms in closely related species are urgently needed to test this hypothesis.

## Taxonomic consequences

Based on the results of our phylogenetic analysis, namely that *Karoowia* is polyphyletic and nested in different clades of

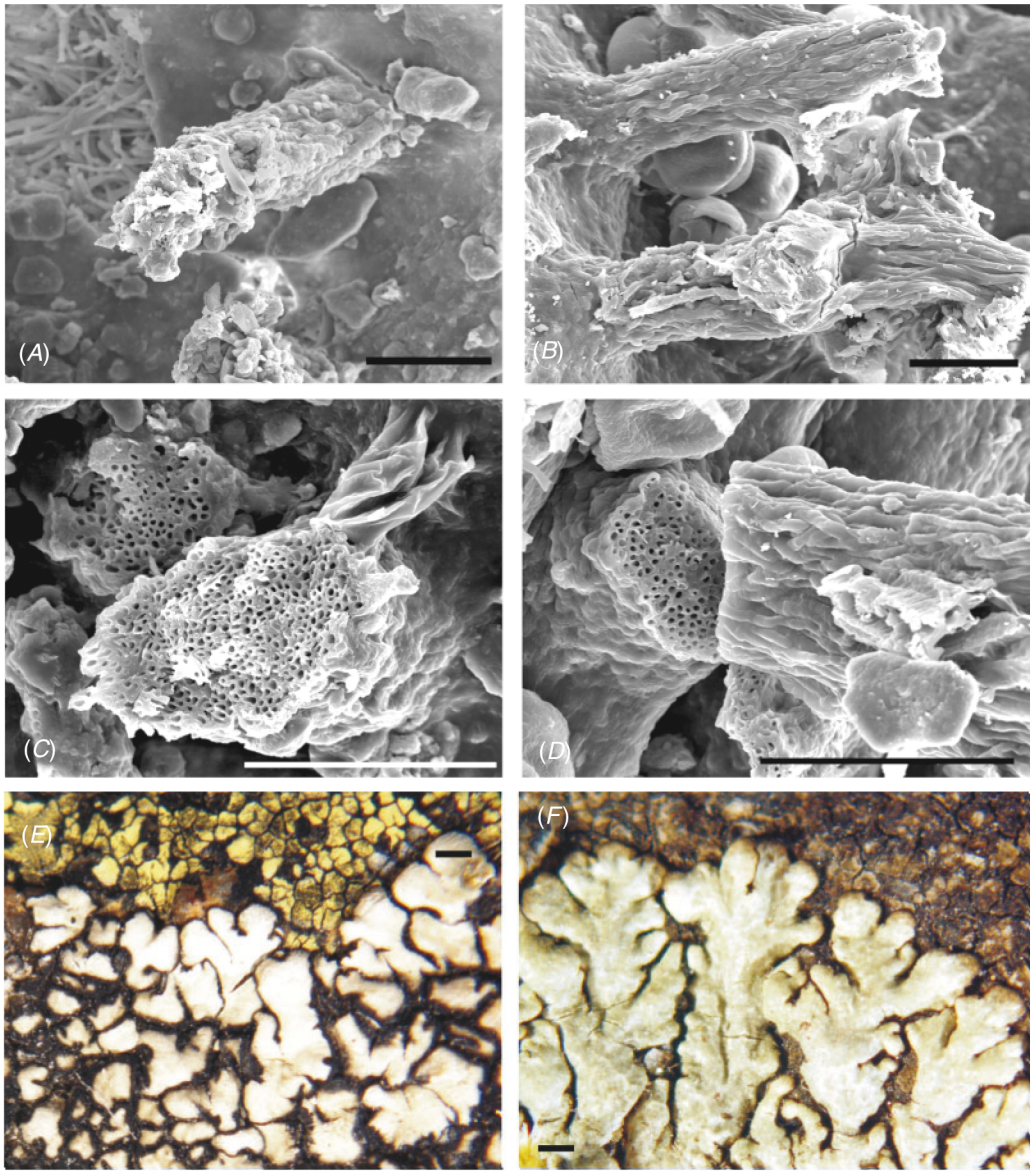


**Fig. 2.** SEM images of the morphology and anatomy of the rhizines of *Karoowia perspersa* (*A*, *C*, *E*) and *Xanthoparmelia mougeotii* (*B*, *D*, *F*). (*A*, *B*) Rhizine surfaces. (*C*, *D*) Rhizine cross-sections. (*E*, *F*) Thallus habits. Scale bars: (*A*, *B*) = 50 µm, (*C*, *D*) = 60 µm, (*E*, *F*) = 0.5 mm.

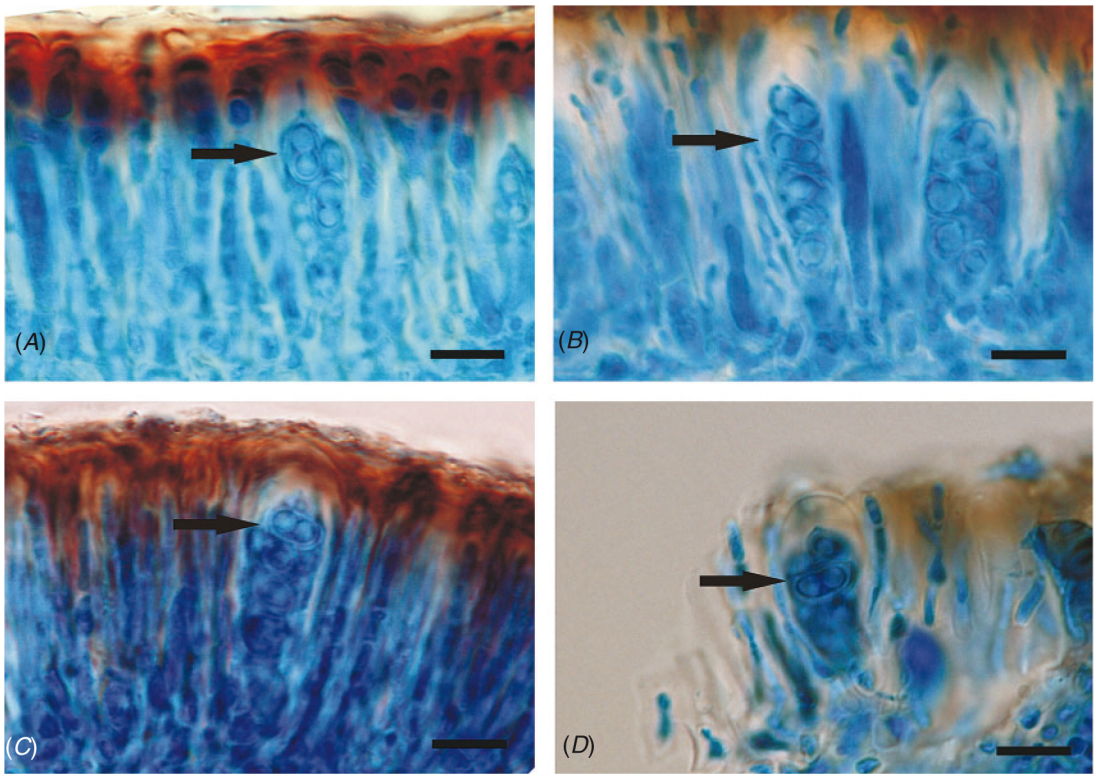


**Fig. 3.** Ascospores of species in the *Xanthoparmelia*-clade. (*A*) *Karoowia perspersa*, (*B*) *Karoowia scitula*, (*C*) *Karoowia ralla*, (*D*) *Xanthoparmelia greytonensis*. Scale bars = 10 µm.

**Table 3. Frequencies of occurrence of conidial types in correlation with the growth form in species of the *Xanthoparmelia*-clade (including *Karoowia* spp.)**

Based on literature (Elix 1994, 2001; Esslinger 1977; Hale 1990; Nash *et al.* 1995; Nash and Elix 2004)

| Conidia type | Thallus type | | |
|---|---|---|---|
| | Subcrustose | | Foliose |
| | *Karoowia* | *Xanthoparmelia* | *Xanthoparmelia* |
| Cylindrical-Filiform | 16 (84.2%) | 14 (19.8%) | 7 (2%) |
| Intermediate (Bacilliform to weakly bifusiform) | 3 (15.8%) | 19 (26.8%) | 65 (18.8%) |
| Short bifusiform | 0 (0%) | 38 (53.6%) | 273 (79.2%) |
| Total species | 19 | 71 | 345 |

*Xanthoparmelia*, we formally reduce *Karoowia* to synonymy with *Xanthoparmelia*. This necessitates fifteen new combinations in *Xanthoparmelia* which are made below. One new name is also proposed. Three *Karoowia* species already have combinations in *Xanthoparmelia*.

***Xanthoparmelia*** (Vain.) Hale, Phytologia 28: 485 (1974)

*Type*: *Xanthoparmelia conspersa* (Ehrh. ex Ach.) Hale, Phytologia 28: 485 (1974). ≡ *Lichen conspersus* Ehrh. ex Ach., Lichenogr. Suec. Prodr.: 118 (1799 '1798').

*Syn.*: *Karoowia* Hale, Mycotaxon 35: 182 (1989).

***Xanthoparmelia adligans*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia adligans* Brusse, Mycotaxon 31: 533 (1988). ≡ *Karoowia adligans* (Brusse) Hale, Mycotaxon 35: 184 (1989).

***Xanthoparmelia arquata*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia arquata* Brusse, Mycotaxon 31: 535 (1988). ≡ *Karoowia arquata* (Brusse) Hale, Mycotaxon 35: 184 (1989).

***Xanthoparmelia ganymedea*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia ganymedea* Brusse, Mycotaxon 31: 536 (1988). ≡ *Karoowia ganymedea* (Brusse) Hale, Mycotaxon 35: 185 (1989).

***Xanthoparmelia insipida*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia insipida* Brusse, Mycotaxon 27: 238 (1986). ≡ *Karoowia insipida* (Brusse) Hale, Mycotaxon 35: 185 (1989).

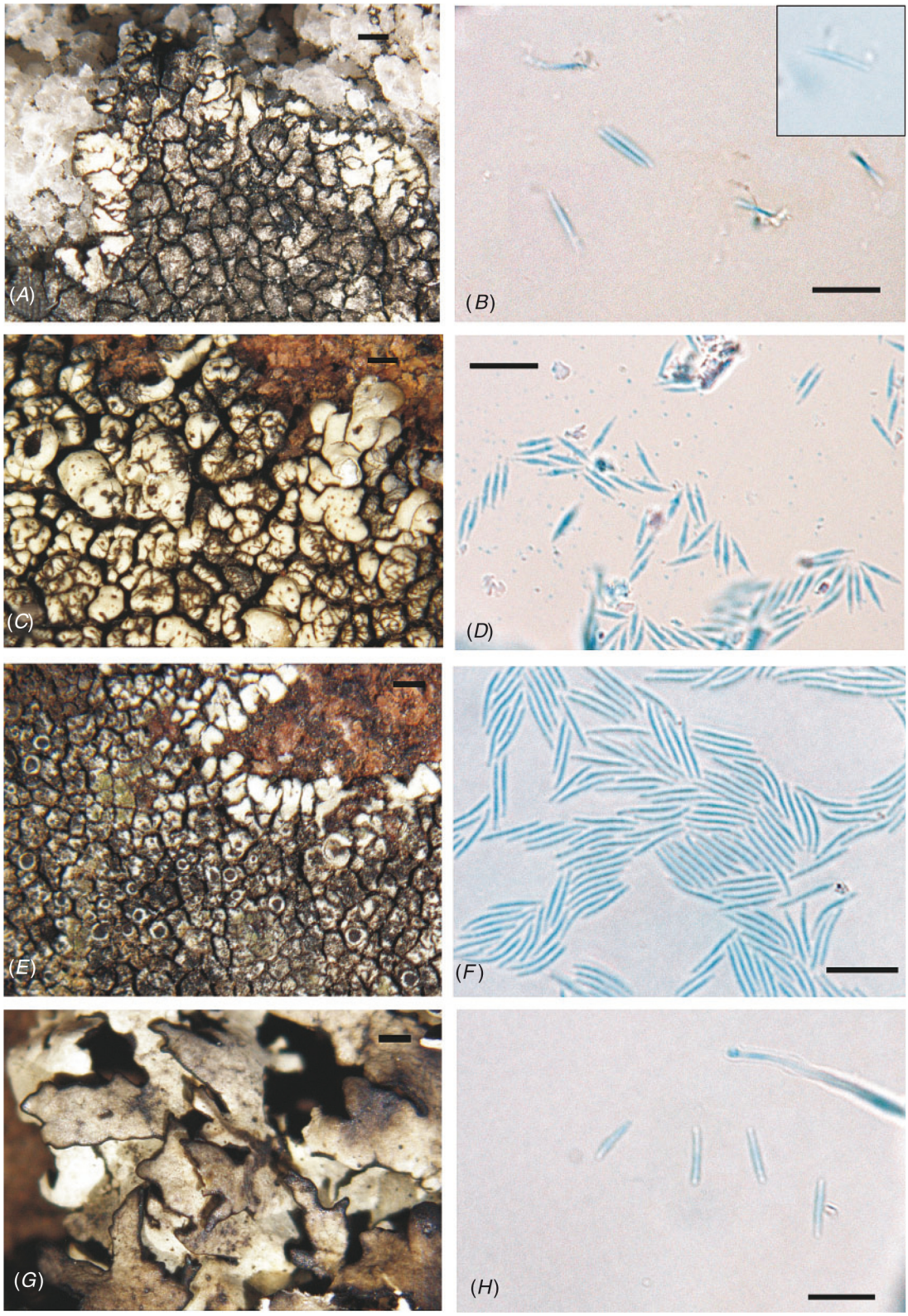


**Fig. 4.** Thallus morphology and conidia of species in the *Xanthoparmelia*-clade. (*A*) *Karoowia adhaerens*, habit, (*B*) cylindrical conidia. (*C*) *Karoowia scitula*, habit, (*D*) bifusiform conidia. (*E*) *Karoowia ralla*, habit, (*F*) filiform conidia. (*G*) *Xanthoparmelia tegeta*, habit, (*H*) cylindrical conidia. Scale bar for habit photos = 0.5 mm and for microscopic pictures = 10 µm.

**Table 4. Analysis of the cell wall polysaccharide-types following Common (1991)**

| Species | Polysaccharide-type |
|---|---|
| *Flavoparmelia caperata* (MAF6045) | Isolichenan-type |
| *Parmotrema hypoleucinum* (MAF1135–1) | Parmotrema-type |
| *Xanthoparmelia mougeotii* (MAF6802) | Xanthoparmelia-type |
| *Xanthoparmelia greytonensis* (MAF16210) | Xanthoparmelia-type |
| *Karoowia adhaerens* (MAF16212) | Xanthoparmelia-type |
| *Karoowia perspersa* (MAF15538) | Xanthoparmelia-type |
| *Karoowia ralla* (MAF15537) | Xanthoparmelia-type |
| *Karoowia saxeti* (MAF16224) | Xanthoparmelia-type |
| *Karoowia scitula* (MAF15536) | Xanthoparmelia-type |
| *Karoowia subchalybaeizans* (MAF15533) | Xanthoparmelia-type |
| *Karoowia* aff. *supposita* (MAF16206–1) | Xanthoparmelia-type |

***Xanthoparmelia leptoplaca*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia leptoplaca* Brusse, Mycotaxon 27: 242 (1986). ≡ *Karoowia leptoplaca* (Brusse) Hale, Mycotaxon 35: 185 (1989).

***Xanthoparmelia lyrigera*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia lyrigera* Brusse, Mycotaxon 35: 24 (1989). ≡ *Karoowia lyrigera* (Brusse) Elix, Mycotaxon 70: 108 (1999). ≡ *Paraparmelia lyrigera* (Brusse) Elix, Mycotaxon 63: 341 (1997).

***Xanthoparmelia microscopica*** (Hale) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Karoowia microscopica* Hale, Mycotaxon 35: 187 (1989).

***Xanthoparmelia mucinae*** Amo, A.Crespo, Elix & Lumbsch, *nom. nov.*

*Pro*: *Parmelia squamatica* Brusse, Mycotaxon 27: 242 (1986). ≡ *Karoowia squamatica* (Brusse) Hale, Mycotaxon 35: 192 (1989); non *Xanthoparmelia squamatica* Elix & T.H. Nash, Mycotaxon 73: 60 (1999).

*Etymology*

The new name for this species is chosen to honour our friend and colleague Ladislav Mucina, vegetation scientist and former Professor of Botany at the University of Stellenbosch in South Africa.

***Xanthoparmelia perspersa*** (Stizenb.) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia perspersa* Stizenb., Verh. Thätigk. St. Gall. Naturw. Ges. 1888–1889: 152 (1890). ≡ *Karoowia perspersa* (Stizenb.) Hale, Mycotaxon 35: 187 (1989).

***Xanthoparmelia protocetrarica*** (Hale) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Karoowia protocetrarica* Hale, Mycotaxon 35: 187 (1989).

***Xanthoparmelia ralla*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia ralla* Brusse, Mycotaxon 27: 240 (1986). ≡ *Karoowia ralla* (Brusse) Hale, Mycotaxon 35: 189 (1989).

***Xanthoparmelia salazinica*** (Hale) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Karoowia salazinica* Hale, Mycotaxon 35: 189 (1989).

***Xanthoparmelia saxeti*** (Stizenb.) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia saxeti* Stizenb., Verh. Thätigk. St. Gall. Naturw. Ges. 1888–1889: 153 (1890). ≡ *Karoowia saxeti* (Stizenb.) Hale, Mycotaxon 35: 190 (1989).

***Xanthoparmelia scitula*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia scitula* Brusse, Bothalia 15: 137 (1984). ≡ *Karoowia scitula* (Brusse) Hale, Mycotaxon 35: 190 (1989).

***Xanthoparmelia spissa*** (Brusse) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Parmelia spissa* Brusse, Mycotaxon 31: 158 (1988). ≡ *Karoowia spissa* (Brusse) Hale, Mycotaxon 35: 190 (1989).

***Xanthoparmelia subchalybaeizans*** (Hale) Amo, A.Crespo, Elix & Lumbsch, *comb. nov.*

*Bas.*: *Karoowia subchalybaeizans* Hale, Mycotaxon 35: 192 (1989).

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# Origen y diversificación de los principales clados de los líquenes parmelioides (Parmeliaceae, Ascomycota) durante el Paleógeno inferidos mediante análisis Bayesiano

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## Resumen

Existe un largo debate sobre el papel de la vicarianza y la dispersión a larga distancia para explicar la distribución actual de los organismos, especialmente en aquellos con pequeñas diásporas que pueden ser dispersadas potencialmente a larga distancia. La estimación de la edad de los clados es un dato crucial para evaluar el impacto de estos procesos. El objetivo de este estudio es profundizar en el conocimiento de la historia evolutiva del mayor clado de los hongos liquenizados, los líquenes parmelioides (Parmeliaceae, Lecanoromycetes, Ascomycota) mediante la datación del origen del grupo y de sus principales linajes. Estos líquenes presentan una distribución mundial, con centros de diversidad en el Neo- y Paleotrópico y en las regiones semiáridas subtropicales del hemisferio Sur. El análisis filogenético se llevó a cabo usando secuencias de ADN ribosómico del nuLSU y el mtSSU y el gen RPB1 codificante de proteína. Las tres regiones de ADN tienen diferente tasa evolutiva: el gen RPB1 tiene una tasa entre 2 y 4 veces superior al nuLSU y mtSSU. Los tiempos de divergencia de los principales clados parmelioides fueron estimados con un análisis BEAST particionado, permitiendo diferentes tasas evolutivas para cada región de ADN y usando un modelo de reloj evolutivo relajado. Se usaron tres puntos de calibración para datar el árbol: la edad inferida para el origen del linaje de los Lecanoromycetes y dos fósiles datados (un fósil de *Parmelia* en el grupo parmelioide y un fósil de *Alectoria* dentro de la familia Parmeliaceae). La filogenia datada de los líquenes parmelioides se comparó con las condiciones paleoclimáticas y con un cladograma paleogeográfico. Los líquenes parmelioides se diversificaron en torno al límite K/T (Cretácico/Terciario) y los principales clados se originaron entre el Eoceno y el Oligoceno. La radiación de los géneros ocurrió en diferentes condiciones climáticas globales entre el Oligoceno temprano, el Mioceno y el Plioceno temprano. Los tiempos estimados de divergencia de los diferentes grupos de parmelioides apuntan a que el principal factor para explicar los patrones actuales de distribución biogeográfica en los líquenes parmelioides del Hemisferio Sur es la dispersión a larga distancia, especialmente en la disyunciones entre África y Australia, porque la secuencia de fragmentación de Gondwana comenzó antes que el origen de estos clados. Sin embargo, en el caso de las disyunciones entre Australia y Sudamérica nuestros datos no pueden rechazar la vicarianza como explicación de estas disyunciones.

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# Origin and Diversification of Major Clades in Parmelioid Lichens (Parmeliaceae, Ascomycota) during the Paleogene Inferred by Bayesian Analysis

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## Abstract

There is a long-standing debate on the extent of vicariance and long-distance dispersal events to explain the current distribution of organisms, especially in those with small diaspores potentially prone to long-distance dispersal. Age estimates of clades play a crucial role in evaluating the impact of these processes. The aim of this study is to understand the evolutionary history of the largest clade of macrolichens, the parmelioid lichens (Parmeliaceae, Lecanoromycetes, Ascomycota) by dating the origin of the group and its major lineages. They have a worldwide distribution with centers of distribution in the Neo- and Paleotropics, and semi-arid subtropical regions of the Southern Hemisphere. Phylogenetic analyses were performed using DNA sequences of nuLSU and mtSSU rDNA, and the protein-coding *RPB1* gene. The three DNA regions had different evolutionary rates: *RPB1* gave a rate two to four times higher than nuLSU and mtSSU. Divergence times of the major clades were estimated with partitioned BEAST analyses allowing different rates for each DNA region and using a relaxed clock model. Three calibrations points were used to date the tree: an inferred age at the stem of Lecanoromycetes, and two dated fossils: *Parmelia* in the parmelioid group, and *Alectoria*. Palaeoclimatic conditions and the palaeogeological area cladogram were compared to the dated phylogeny of parmelioid. The parmelioid group diversified around the K/T boundary, and the major clades diverged during the Eocene and Oligocene. The radiation of the genera occurred through globally changing climatic condition of the early Oligocene, Miocene and early Pliocene. The estimated divergence times are consistent with long-distance dispersal events being the major factor to explain the biogeographical distribution patterns of Southern Hemisphere parmelioids, especially for Africa-Australia disjunctions, because the sequential break-up of Gondwana started much earlier than the origin of these clades. However, our data cannot reject vicariance to explain South America-Australia disjunctions.

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## Introduction

In traditional, morphology-based concepts, lichenized fungi often have wide distributions spanning over several continents with a number of species being cosmopolitan. This has led to a widespread notion that the distribution of these fungi is primarily shaped by ecological conditions rather than explained by historical events. In contrast, a few authors have invoked plate tectonics and emphasized vicariance to explain distribution patterns of lichens, especially for species occurring in the Southern Hemisphere [1–6]. However, within the last decade, molecular data have helped to revolutionize the species delimitation of lichenized fungi and demonstrated that morphology-based concepts largely underestimate the diversity of lichens [7–16]. As a result of these studies, it became clear that – although the number of widely distributed species in lichenized fungi is generally higher than in plants or animals – lichens have more restricted distribution areas than previously assumed. For example, supposedly cosmopolitan species in Parmeliaceae and Physciaceae were found to represent distinct lineages in different continents [11,13]. Further, recent progress in our knowledge of the phylogeny of some clades of lichenized fungi revealed the presence of clades at generic rank that originated and diversified in the Southern Hemisphere [17–21]. Hence, we have turned our attention to address the issue of the extent of vicariance and long-distance dispersal to understand the current distribution of lichenized fungi anew using molecular phylogenies.

For this purpose a dated phylogeny with the estimated ages of origin and diversification of the parmelioid group is required. A main problem for building dated phylogenies in fungi is the poor fossil record. While our understanding of the divergence time of angiosperms is well established [22–25],. time estimates for fungi were long disputed based on uncertainties in the interpretation of the few known fossils [26]. Consequently, published dating estimates ranged from 660 million to 2.15 billion years ago for the origin of Fungi and from 390 million to 1.5 billion years for the split of the two crown groups of fungi, Ascomycota and Basidiomycota [27–31]. Re-examination of the morphology of

the fossils and re-evaluation of the published dating studies, however, suggested more consistent results with the origin of the Fungi dating back to between 760 million and 1.06 billion years, and the split of Ascomycota and Basidiomycota for about 500–650 Ma [26,32]. This suggests that terrestrial fungi evolved and diversified more or less simultaneously with the evolution of land plants.

Given that the phylogeny of parmelioid lichens is well resolved [18] and the recent advantages in our understanding of the timing of major events in the evolution of fungi, we feel confident that the times of main nodes differentiation can be estimated. Moreover, this dating approach can be applied to address the issue of the extent of vicariance on distribution patterns in lichens and use parmelioids as an example.

The family Parmeliaceae is widely distributed throughout the world from polar to tropical regions and is one of the largest families of lichenized Ascomycota [18,33–36]. Most species form foliose or fruticose thalli, but some also have subcrustose, umbilicate, peltate thalli, and even lichenicolous fungi were found to belong here [18,35,37]. The family includes about 2500 species classified in 84 genera and is characterized by cup-shaped apothecia with cupulate exciple, *Lecanora*-type asci, often with hyaline ascospores [35]. Within the family, six strongly supported major monophyletic groups can be distinguished [35], which are: alectorioid, cetrarioid, hypogymnioid, letharioid, parmelioid and psiloparmelioid. By far, the largest of these groups is the parmelioid group with about 1500 species [38,39] classified in 27 accepted genera [18]. Phenotypically, the parmelioid lichens are characterized by having mostly foliose thalli with thread-like rhizines on the lower surface, cup-shaped apothecia on the thallus upper surface and *Lecanora*-type asci with hyaline ascospores (Fig. 1). Within the parmelioid lichens, eight major monophyletic clades can be distinguished, which are *Cetrelia*-, *Hypotrachyna*-, *Melanohalea*-, *Parmelia*-, *Parmelina*-, *Parmeliopsis*-, *Parmotrema*- and *Xanthoparmelia*-groups [18]. During the last decades, phylogenetic studies based on DNA sequence data have greatly advanced our understanding of the evolution of the family including the phylogenetic relationships among major clades [18,34–35,40–46], phenotypical evolution [35,45,47], disparity in substitution rates among clades [48], and the geographic origin of certain clades [17,19]. However, so far none of these have aimed at dating major cladogenesis events in Parmeliaceae. Besides the issues discussed above, this is probably also due to the poor fossil record of Parmeliaceae. Only very few fossils have been recorded of Parmeliaceae and the known ones are all preserved in amber. An *Alectoria* species was described from Baltic amber (35–40 Ma) [49].

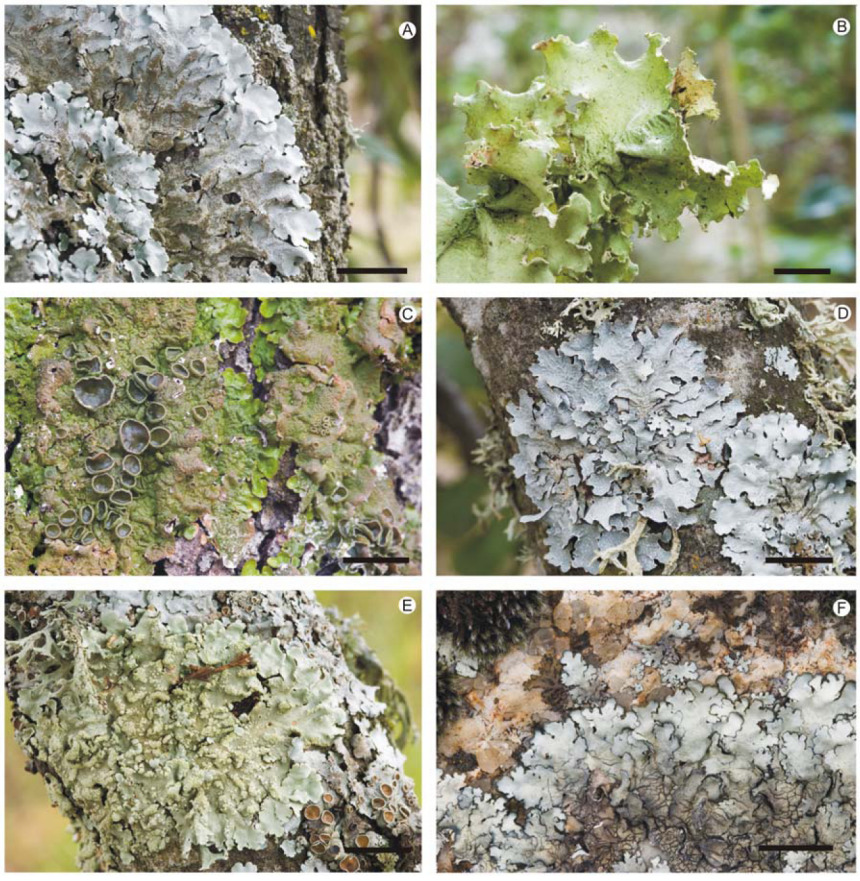


**Figure 1. Selected examples of different genera and species of parmelioid lichens.** A. *Parmelina tiliacea*, one of the most common lichens in the Mediterranean basin. B. *Parmotrema hypoleucinum*, endemic of the southwestern Mediterranean Region, occurring in warm and humid areas. C. *Melanelixia glabra* occurs from southwestern Europe to eastern Russia. D. *Parmelia sulcata*, a common species from cold to temperate regions of both Hemispheres. E. *Flavoparmelia soredians* occurs in warm and humid areas of temperate regions of both Hemispheres. F. *Xanthoparmelia conspersa*, one of the most widespread species of macrolichens growing on acid rocks in temperate areas of both Hemipheres, excluding Australia and South Africa. The distribution areas after Nimis [105]. All photographs were taken in the field. Scale = 1 cm.
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Two fossil species of *Parmelia* were described from Dominican amber (15–45 Ma) [50] and two specimens of *Anzia* have been found from European amber (35–40 Ma) [51].

The aims of this study are 1) to put a time-scale on the phylogenetic tree of parmelioid lichens and thus identify when the main nodes differentiated, employing calibration points from a recent dating estimate of fungi [26] and available fossil reference-points, and 2) to address the impact of vicariance and long-distance dispersal processes in the distribution patterns in parmelioid lichens. Specifically we address the question whether plate tectonics can be invoked to explain distribution areas of species groups or species occurring in the Southern Hemisphere.

## Results

### Data and substitution patterns

For the analyses a dataset of three loci of 225 OTUs was used. The matrix included 1849 unambiguously aligned nucleotide characters, with 599 positions in the *RPB1*gene, 655 positions in the nuLSU and 595 positions in the mtSSU. The number of constant characters was 643. The likelihood value of the ML tree obtained with Garli was lnL = −59525.412. The constrained position of Chaethothyriomycetidae as sister group of Lecanoromycetes is not significantly worse than the unconstrained topology (*p*-SH = 0.444, *c*-ELW = 0.441).

The substitution rates of the three loci (nuLSU, mtSSU and RPB1) are shown for each lineage of the parmelioid lichens in Fig. 2 and Table 1. The different lineages have similar range of variation in each gene but there are clear differences in mean rates between the three genes, the rate of *RPB1* being two to four times higher than mtSSU and nuLSU, respectively.

### Estimating divergence times

A chronogram based on the analysis of the combined matrix of three loci is shown in Fig. 3, showing the relationships of Lecanoromycetes and Parmeliaceae (highlighted in dark grey color). The detailed chronogram for Parmeliaceae is depicted in Fig. 4. This analysis used as calibration points the age of the split of Lecanoromycetes-Chaethothyriomycetidae (C1); the age of the fossil (*P. ambra*) assigned to the *Parmelia* s.s. crown node (C2), and the age of the fossil *A. succini* assigned to the *Alectoria* crown (C3). The node ages, 95% highest posterior density intervals (HPD) of ages, substitution rates and 95% HPD of substitution rates for the main clades, divergence points and diversification point of the genera are shown in Table 2.

The alternative analyses gave similar results (Table 3). The small differences are as follows:1) using two calibration points (C1 and C2) and excluding the *Alectoria* fossil, node ages and 95% HPD intervals are slightly younger for some of the parmelioid clades; 2) using C1 and C3 and excluding *Parmelia*, the node ages and 95% HPD intervals had a small decrease in older clades; and 3) using C1, C2 and the *Alectoria* fossil constraining the age of the alectorioid clade (*Alectoria*, *Bryoria* and *Pseudephebe*; C3*) the node ages are similar with very small fluctuations. The 95% HPD intervals for all the clades largely agree and are similar to those obtained in the first analysis using the calibration points C1, C2 and C3.

The split of the Parmeliaceae core was estimated to be around 109 Ma (85.52–136.55 Ma) when the crustose genus *Protoparmelia* separated from the rest of the Parmeliaceae (Fig. 4, Table 2). The basal radiation of Parmeliaceae core took place between 60 and 74 Ma, when the main lineages of the family originated. The radiation of the large group of Parmelioid lichens was estimated to have begun in the Paleocene, around 60 Ma (49.81–73.55 Ma). The morphologically close but phylogenetically distant cetrarioid group, mostly distributed in temperate to alpine regions, was estimated to radiate in the Eocene (27 Ma, 18.81–37.90 Ma, Fig. 4) while the alectorioid clade radiated about 47 Ma (40.88–54.97 Ma).

Our analyses suggest seven separate major divergence events that led to the evolution of the main clades of parmelioid lichens (Fig. 4, marked with dots and numbers). The earliest divergence,

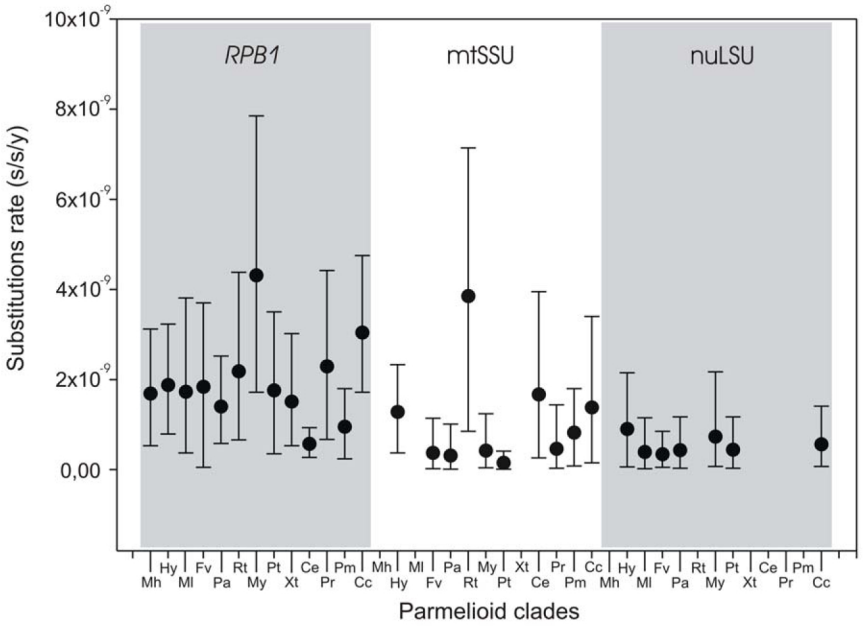


**Figure 2. Substitution rates for the three loci (mtSSU, nuLSU, *RPB1*) of the main clades in Parmelioid.** Dots represent mean rates and bars cover the 95% highest posterior density (HPD). Units: substitution/site/year. Abbreviations for the Parmelioid clades are listed in Table 1.
doi:10.1371/journal.pone.0028161.g002

**Table 1.** Substitution rates of the main clades of parmelioid lichens obtained in the independent BEAST analyses for each locus.

| | RPB1 | | mtSSU | | nuLSU | |
|---|---|---|---|---|---|---|
| **Node MRCA** | **Rate** | **Rate 95% HPD** | **Rate** | **Rate 95% HPD** | **Rate** | **Rate 95% HPD** |
| Lecanoromycetes | 2.01 | 0.37–4.89 | 0.67 | 0.01–1.80 | 0.28 | 0.02–0.77 |
| Lecanoromycetidae | 1.68 | 0.25–3.75 | 0.51 | 0.02–1.31 | - | - |
| Lecanorales | 1.65 | 0.39–3.26 | 0.84 | 0.09–1.89 | 0.71 | 0.06–1.84 |
| Parmeliaceae (*Protoparmelia* included) | 1.85 | 0.49–3.83 | - | - | 1.12 | 0.23–2.20 |
| Parmeliaceae (*Protoparmelia* excluded) | 2.57 | 1.13–4.63 | 0.69 | 0.08–1.66 | 0.70 | 0.13–1.70 |
| parmelioid group | 2.58 | 0.43–5.01 | - | - | - | - |
| *Melanohalea* (Mh) | 1.69 | 0.53–3.12 | - | - | - | - |
| Hypotrachyna-clade (Hy) | 1.88 | 0.79–3.23 | 1.28 | 0.37–2.33 | 0.90 | 0.06–2.15 |
| *Melanelixia* (Ml) | 1.73 | 0.37–3.81 | - | - | 0.39 | 0.02–1.15 |
| *Flavoparmelia* (Fv) | 1.84 | 0.05–3.70 | 0.37 | 0.02–1.14 | 0.34 | 0.05–0.85 |
| *Parmelia* (Pa) | 1.40 | 0.58–2.52 | 0.31 | 0.01–1.01 | 0.43 | 0.03–1.17 |
| *Remototrachyna* (Rt) | 2.18 | 0.66–4.38 | 3.85 | 0.85–7.14 | - | - |
| *Myelochroa* (My) | 4.31 | 1.72–7.85 | 0.42 | 0.04–1.24 | 0.73 | 0.07–2.17 |
| *Punctelia* (Pt) | 1.76 | 0.35–3.50 | 0.15 | 0.01–0.41 | 0.44 | 0.03–1.17 |
| *Xanthoparmelia* (Xt) | 1.51 | 0.53–3.02 | - | - | - | - |
| *Cetrelia* (Ce) | 0.57 | 0.27–0.93 | 1.67 | 0.26–3.95 | - | - |
| *Parmotrema* (Pr) | 2.29 | 0.67–4.42 | 0.46 | 0.03–1.44 | - | - |
| *Parmelina* (Pm) | 0.95 | 0.24–1.80 | 0.82 | 0.08–1.80 | - | - |
| Canoparmelia crozalsiana-clade (Cc) | 3.04 | 1.72–4.75 | 1.38 | 0.15–3.40 | 0.56 | 0.07–1.41 |

Units: substitution/site/year $\times 10^{-9}$. HPD: highest posterior density interval.
doi:10.1371/journal.pone.0028161.t001

estimated around 48 Ma, separated the *Xanthoparmelia*-clade from the *Parmotrema*-clade (including the genera *Austroparmelina*, *Canoparmelia*, *Flavoparmelia*, *Punctelia*, *Flavopunctelia*, *Nesolechia* and *Parmotrema*). In subsequent divergence events during Eocene time *Melanohalea* split from *Melanelixia* (about 42 Ma), *Parmelina* separated from *Myelochroa* (37 Ma), *Remototrachyna* from *Bulbothrix* (about 37 Ma) and *Austroparmelina* differentiated from the most recent common ancestor (MRCA) of *Parmotrema*, *Flavoparmelia* and *Canoparmelia crozalsiana*-clade (about 34 Ma). During the early Oligocene *Cetrariastrum* (about 32 Ma) differentiated from the rest of the complex *Hypotrachyna*-clade (including *Everniastrum*, *Hypotrachyna* s.l., and *Parmelinopsis*), and. *Flavoparmelia* separated from *Parmotrema* and *Canoparmelia crozalsiana*-clade (30 Ma).

Our data indicate that diversification of the recent genera of parmelioid lichens occurred during Oligocene and Miocene. The diversification of the *Melanelixia* was estimated to be around (34 Ma), and in a second radiation event (22–25 Ma) diversified *Melanohalea*, *Parmelia*, *Remototrachyna*, *Flavoparmelia* and *Xanthoparmelia*. *Myelochroa*, *Punctelia* and *Parmotrema* radiated during the Miocene (between 14 and 18 Ma), and *Cetrelia*, *Parmelina* and *Canoparmelia crozalsiana*-clade was estimated to radiated by late Miocene and early Pliocene (4–9 Ma).

The estimated ages of diversification of the main clades of parmelioid lichens compared to the geological area cladogram representing the relationships among the Southern Hemisphere landmasses are shown in Figure 5. The break-up of the main landmasses of the South Hemisphere predate the ages of diversification of the parmelioid clades and the splits of the main lineages (Fig. 5). The presence of several genera (e.g. *Xanthoparmelia*, *Parmotrema*, *Bulbothrix*, *Austroparmelina*, *Flavoparmelia*, *Melanelixia*, *Myelochroa*, *Hypotrachyna*, *Punctelia*) in different continents of the Southern Hemisphere (A and B, Fig. 5) cannot be explained by continental drift and vicariance because when the parmelioid lineages started to diverge (60 Ma) Africa had already separated from South America, India, New Zealand and Australia.

## Discussion

In this study we put a time-scale on the phylogenetic tree of parmelioid lichens using three DNA regions with different evolutionary rates. The estimated ages allow addressing how vicariance and long-distance dispersal shaped the current distribution patterns of parmelioid lichens, specially the disjunct distributions of species groups in the Southern Hemisphere. Moreover the dated phylogeny provides a general picture of the palaeoclimatic conditions prevalent on Earth when the main lineages differentiated.

The three DNA regions used in this study to build the phylogenetic trees have different evolutionary rates. We found higher substitution rates in the protein coding gene *RPB1* than in the nuLSU and mtSSU ribosomal DNA (Fig. 2, Table 2). Ribosomal DNA has been frequently used in molecular studies of Parmeliaceae and other lichenized fungi [34,41–42,44–47,52–54] but so far few molecular studies have used protein-coding genes to infer phylogenetic relationships in Parmeliaceae [18,35,43,55–56]. In our analysis the *RPB1* gene (with high substitution rates) provided better resolution of the terminal lineages of the tree while the more conserved genes with lower substitution rate (nuLSU, mtSSU) better supported the backbone of the tree topology.

Substitution rates have been used to estimate divergence times due to the lack of fossil records. Takamatsu & Matsuda [57] calculated a substitution rates for Erysiphales ($2.52\times10^{-9}$ s/s/y for nuLSU). Our estimation of the substitution rate for the nuLSU of the Parmeliaceae ($1.12\times10^{-9}$ s/s/y; Table 2) is in the same

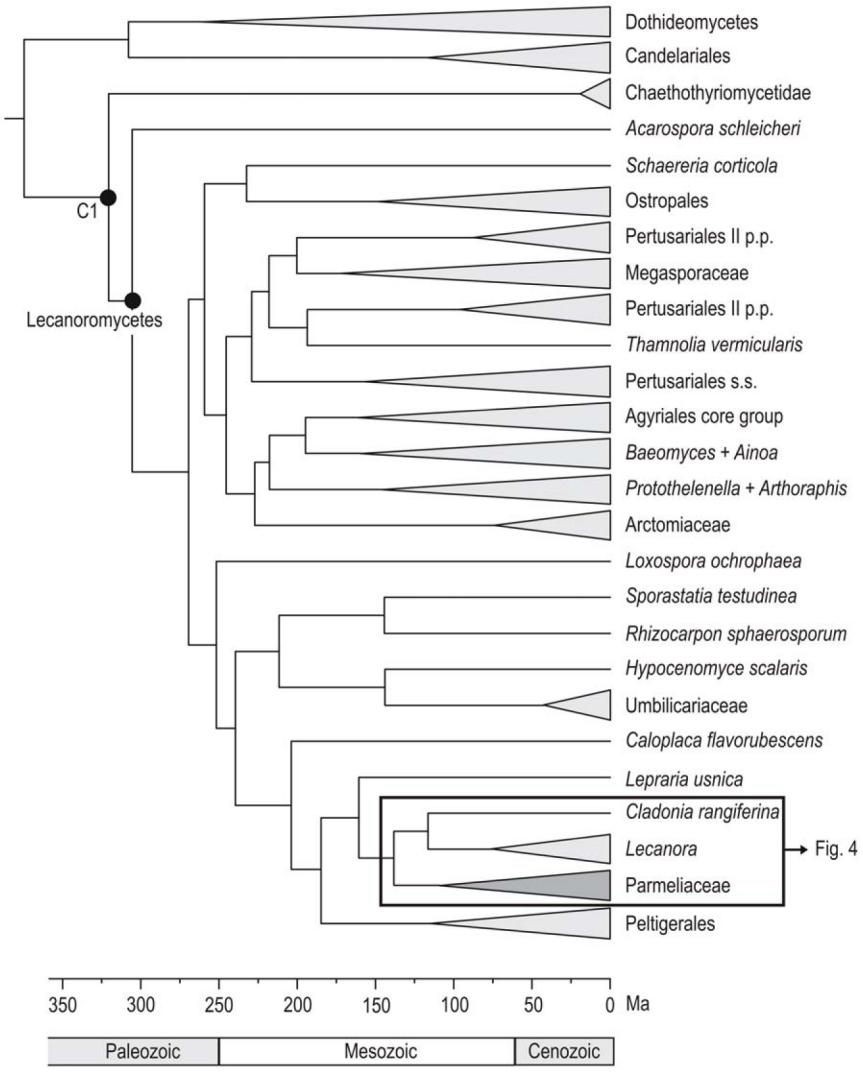


**Figure 3. Lecanoromycetes tree indicating the position of the detailed chronogram of Parmeliaceae shown in Figure 4.** The chronogram was estimated from a partitioned data set of three loci (mtSSU, nuLSU, *RPB1*) using BEAST. The calibration point (C1) was set at the divergence node of Lecanoromycetes and Chaethothyriomycetidae.
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order of magnitude but slower than in the phytopathogenous fungi of Erysiphales. This indicates that caution should be used when applying substitution rates of a group of fungi to estimate divergence times of an unrelated group [58–59].

The scarcity and uncertainty of the fossil record was a major obstacle for estimation of dates of radiation in most groups of fungi. We have estimated divergence times of the major lineages of Parmeliaceae and times of the main radiations of the genera using a comprehensive phylogenetic hypothesis of the family, calibrated with the available fossil evidence, a root time inferred from [26], and allowing a relaxed-clock model for the rates of evolution of the main clades.

Berbee & Taylor [32] estimated the age of the parmeliaceae crown node at about 60 Ma based on substitution rates, and a minimum age of the family was given at about 40 Ma according to fossil records [50–51]. However, the age estimates obtained herein suggested that Parmeliaceae evolved much earlier. Our analyses indicate that the Parmeliaceae core originated rather recently with a stem node age estimate of 108 Ma (Fig. 3, 4, Table 2) and a crown node age estimate of 74 Ma (Fig. 4, Table 2). For other major families of lichenized ascomycetes much older crown node ages have been estimated (Rivas Plata & Lumbsch, pers. com.), including Graphidaceae (156 Ma), Physciaceae (153 Ma), and Ramalinaceae (126 Ma). The parmelioid clade is the largest and most strongly supported monophyletic group of the family. Within the parmelioid crown a total of seven major divergence events at different times have been found (Fig. 4), ranging from early Eocene (separation of *Xanthoparmelia* from the *Parmotrema*-clade) to Oligocene (separation of *Flavoparmelia* from *Parmotrema* and *Canoparmelia crozalsiana*-clade). The radiations of parmelioid genera were estimated to start at the end of Eocene (*Melanelixia*) and occurred during the Oligocene-Miocene for most of the genera.

Major clades of parmelioid lichens either show distinct distribution patterns of the clade or include numerous species with disjunct distributions, as in the genera *Xanthoparmelia*, *Austroparmelina*, *Melanohalea*, *Parmelina*, and *Remototrachyna* [11,17,19,41,60–64]. Examples of disjunct distributions include *Xanthoparmelia* and *Austroparmelina*. *Xanthoparmelia* with ca. 800 species, the most speciose clade of Parmeliaceae, occurs world-wide, although in some cases the species delimitation has recently

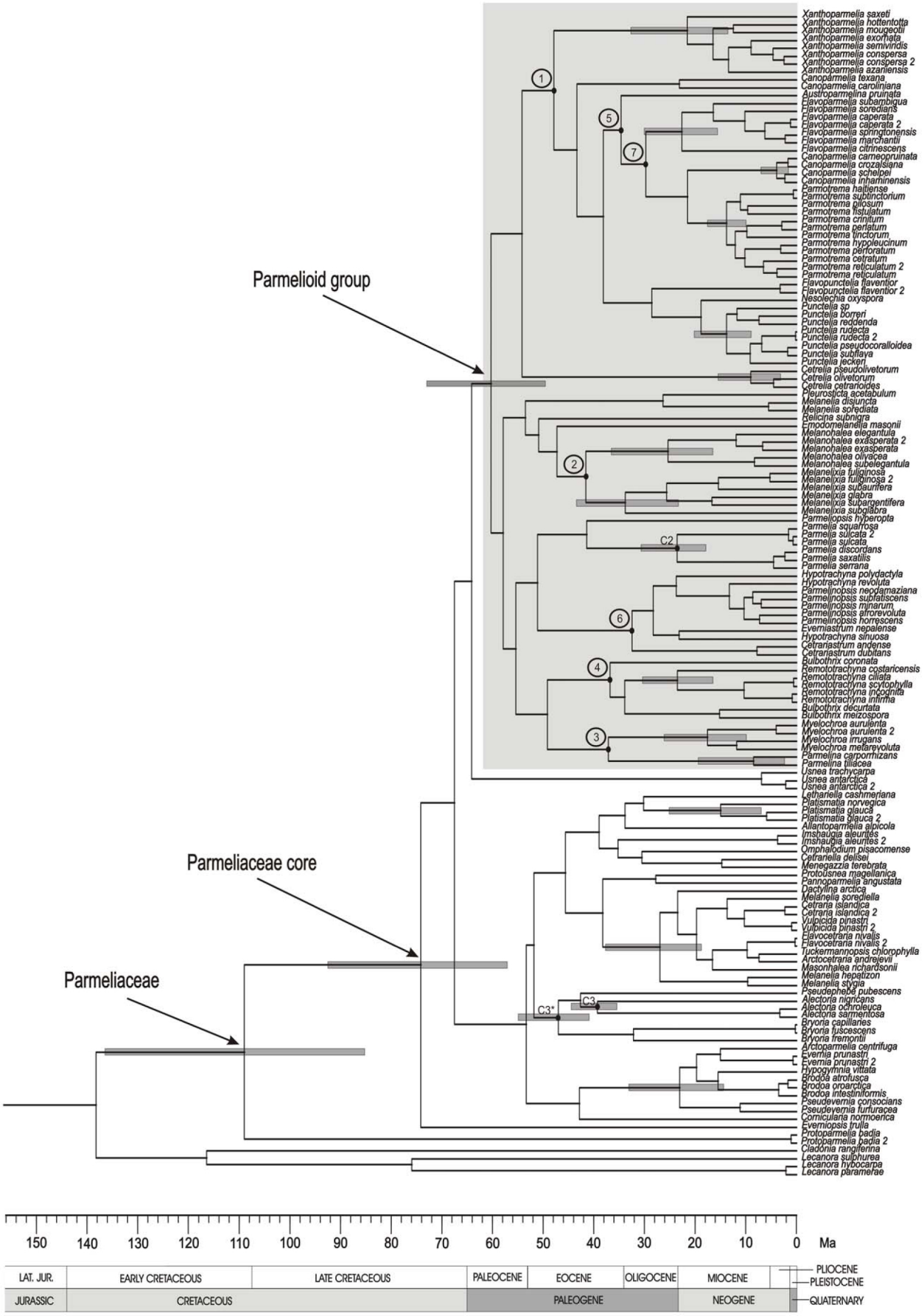
Parmelioid group
Parmeliaceae core
Parmeliaceae
1
2
3
4
5
6
7
C2
C3
C3*
Xanthoparmelia saxeti
Xanthoparmelia hottentotta
Xanthoparmelia mougeotii
Xanthoparmelia exorhata
Xanthoparmelia semiviridis
Xanthoparmelia conspersa
Xanthoparmelia conspersa 2
Xanthoparmelia azaniensis
Canoparmelia texana
Canoparmelia caroliniana
Austroparmelina pruinata
Flavoparmelia subambigua
Flavoparmelia soredians
Flavoparmelia caperata
Flavoparmelia caperata 2
Flavoparmelia springtonensis
Flavoparmelia marchantii
Flavoparmelia citrinescens
Canoparmelia carneopruinata
Canoparmelia crozalsiana
Canoparmelia scheipei
Canoparmelia inharhinensis
Parmotrema haitiense
Parmotrema subtinctorium
Parmotrema pilosum
Parmotrema fistulatum
Parmotrema crinitum
Parmotrema perlatum
Parmotrema tinctorum
Parmotrema hypoleucinum
Parmotrema perforatum
Parmotrema cetratum
Parmotrema reticulatum 2
Parmotrema reticulatum
Flavopunctelia flaventior
Flavopunctelia flaventior 2
Nesolechia oxyspora
Punctelia sp.
Punctelia borreri
Punctelia reddenda
Punctelia rudecta
Punctelia rudecta 2
Punctelia pseudocoralloidea
Punctelia subflava
Punctelia jeckeri
Cetrelia pseudolivetorum
Cetrelia olivetorum
Cetrelia cetrarioides
Pleurosticta acetabulum
Melanelia disjuncta
Melanelia sorediata
Relicina subnigra
Emodomelanelia masonii
Melanohalea elegantula
Melanohalea exasperata 2
Melanohalea exasperata
Melanohalea olivacea
Melanohalea subelegantula
Melanelixia fuliginosa
Melanelixia fuliginosa 2
Melanelixia subaurifera
Melanelixia glabra
Melanelixia subargentifera
Melanelixia subglabra
Parmeliopsis hyperopta
Parmelia squarrosa
Parmelia sulcata 2
Parmelia sulcata
Parmelia discordans
Parmelia saxatilis
Parmelia serrana
Hypotrachyna polydactyla
Hypotrachyna revoluta
Parmelinopsis neodamaziana
Parmelinopsis subfatiscens
Parmelinopsis minarum
Parmelinopsis afrorevoluta
Parmelinopsis horrescens
Everniastrum nepalense
Hypotrachyna sinuosa
Cetrariastrum andense
Cetrariastrum dubitans
Bulbothrix coronata
Remototrachyna costaricensis
Remototrachyna ciliata
Remototrachyna scytophylla
Remototrachyna incognita
Remototrachyna infirma
Bulbothrix decurtata
Bulbothrix meizospora
Myelochroa aurulenta
Myelochroa aurulenta 2
Myelochroa irrugans
Myelochroa metarevoluta
Parmelina carporrhizans
Parmelina tiliacea
Usnea trachycarpa
Usnea antarctica
Usnea antarctica 2
Lethariella cashmeriana
Platismatia norvegica
Platismatia glauca
Platismatia glauca 2
Allantoparmelia alpicola
Imshaugia aleurites
Imshaugia aleurites 2
Omphalodium pisacomense
Cetrariella delisei
Menegazzia terebrata
Protousnea magellanica
Pannoparmelia angustata
Dactylina arctica
Melanelia sorediella
Cetraria islandica
Cetraria islandica 2
Vulpicida pinastri
Vulpicida pinastri 2
Flavocetraria nivalis
Flavocetraria nivalis 2
Tuckermannopsis chlorophylla
Arctocetraria andrejevii
Masonhalea richardsonii
Melanelia hepatizon
Melanelia stygia
Pseudephebe pubescens
Alectoria nigricans
Alectoria ochroleuca
Alectoria sarmentosa
Bryoria capillaris
Bryoria fuscescens
Bryoria tremonitii
Arctoparmelia centrifuga
Evernia prunastri
Evernia prunastri 2
Hypogymnia vittata
Brodoa atrofusca
Brodoa proarctica
Brodoa intestiniformis
Pseudevernia consocians
Pseudevernia furfuracea
Cornicularia normoerica
Evernopsis trulla
Protoparmelia badia
Protoparmelia badia 2
Cladonia rangiferina
Lecanora sulphurea
Lecanora hybocarpa
Lecanora parmeriae
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Ma
LAT. JUR.
EARLY CRETACEOUS
LATE CRETACEOUS
PALEOCENE
EOCENE
OLIGOCENE
MIOCENE
PLIOCENE
PLEISTOCENE
JURASSIC
CRETACEOUS
PALEOGENE
NEOGENE
QUATERNARY

**Figure 4. Divergence time chronogram focusing on Parmeliaceae.** The chronogram was estimated as described in the legend for figure 3. Two calibration points based on fossil assignation are marked as C2 (crown node of *Parmelia*) and C3 (crown node of *Alectoria*). C3* is the alternative calibration with the *Alectoria* fossil assigned to the alectorioid crown. The Parmelioid group is highlighted by the grey box. Numbers inside circles refer to divergence nodes between the main clades. Grey bars show the 95% highest posterior density intervals (HPD). Detailed ages are given in Table 2.
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been challenged [65], and in other cases they have been shown much more restricted than previously known [61–64]. Despite being cosmopolitan, *Xanthoparmelia* has two main areas of distribution in arid regions of the Southern Hemisphere (Australia and Africa). A distribution pattern spanning over Australia and Africa cannot be explained by vicariance since these landmasses separated much earlier (Fig. 5; 110–135 Ma) than the split of the *Xanthoparmelia* lineage from the *Parmotrema*-clade (37–58 Ma). The data, however, cannot reject vicariance as the reason for distribution ranges of species occurring in South America and Australia. The separation of these continents (35–52 Ma) happened at a similar time as the origin of this lineage.

*Austroparmelina* includes 13 species that occur in southern and eastern Australia, Tasmania and New Zealand. Two species have wider distribution areas, occurring also in South Africa (*A. labrosa*, *A. pseudorelicina*; [18]); and one species also is known from South America (*A. labrosa*, Chile; [66]). According to our estimates the genus separated from its sister lineages in the late Eocene. Thus the presence of *Austroparmelina* in South Africa is most plausibly explained as a result of long distance dispersal because separation of Africa took place much earlier (Fig. 5). As in the case of *Xanthoparmelia*, our data cannot reject vicariance events as an explanation for the presence of *A. labrosa* in South America and Australia.

In general the estimated ages of diversification events found in our analyses indicate that disjunct distribution patterns of Southern Hemisphere lineages cannot be explained by vicariance. Transoceanic long distance dispersal is the most plausible cause to explain these distribution patterns. This is especially true for taxa occurring in Africa and Australia. This is consistent with recent studies employing molecular clock approaches interpreting distribution patterns in other groups of fungi and plants [58–59,67–76], suggesting that long-distance dispersal is an important factor to explain the current distribution patterns of plants and

**Table 2.** Estimated ages and substitution rates of the most recent common ancestors (MRCA) for the main clades obtained with partitioned BEAST analyses using three calibration points.

| Period (Epoch) Age | Node MRCA | Age | Height 95% HPD | Rate | Rate 95% HPD |
|---|---|---|---|---|---|
| Carboniferous 360–300 | Lecanoromycetes | 305.53 | 275.46–327.36 | 2.64 | 0.19–6.85 |
| Permian 300–250 | Ostropomycetidae | 259.33 | 221.98–293.64 | 1.31 | 0.19–3.28 |
| | Lecanoromycetidae | 251. 68 | 211.58–284.79 | 1.45 | 0.27–3.17 |
| Jurassic 205–135 | Lecanorales | 160.65 | 129.66–192.63 | 1.40 | 0.18–2.98 |
| Cretaceous 135–65 | Parmeliaceae (*Protoparmelia* included) | 108.96 | 85.52–136.55 | 2.07 | 0.52–4.21 |
| | Parmeliaceae core (*Protoparmelia* excluded) | 74.17 | 57.59–92.56 | 2.92 | 1.29–4.60 |
| Paleogene (Paleocene) 65–53 | Parmelioid group | 60.28 | 49.81–73.55 | 2.87 | 0.71–5.78 |
| Paleogene (Eocene) 53–34 | Split 1. *Xanthoparmelia* – Parmotrema-clade | 47.87 | 37.28–58.49 | 1.64 | 0.24–3.51 |
| | Split 2. *Melanelixia* - *Melanohalea* | 41.55 | 31.25–51.94 | 1.60 | 0.27–3.54 |
| | Split 3. *Parmelina* - *Myelochroa* | 37.13 | 24.97–48.65 | 1.80 | 0.17–4.71 |
| | Split 4. *Remototrachyna* - *Bulbotrix* | 36.78 | 27.66–45.72 | 3.81 | 1.49–6.58 |
| | Split 5. *Austroparmelina*– *Parmotrema, Flavoparmelia, …* | 34.60 | 26.67–42.69 | 1.41 | 0.13–3.08 |
| Paleogene (Oligocene) 34–23.5 | *Melanelixia* | 33.82 | 23.66–43.86 | 1.50 | 0.28–3.26 |
| | Split 6. *Cetrariastrum* - *Hypotrachyna*-clade p.p. | 32.48 | 22.70–43.70 | 2.10 | 0.94–3.61 |
| | Split 7. *Flavoparmelia* - *Parmotrema* | 29.73 | 22.30–36.18 | 2.78 | 0.66–6.64 |
| | *Melanohalea* | 25.38 | 16.58–36.73 | 1.78 | 0.61–3.53 |
| | *Parmelia* | 23.59 | 18.03–31.31 | 1.56 | 0.49–3.10 |
| | *Remototrachyna* | 23.54 | 16.69–30.68 | 1.50 | 0.38–2.78 |
| Neogene (Miocene) 23.5–5.3 | *Flavoparmelia* | 22.61 | 15.71–30.09 | 2.25 | 0.66–4.48 |
| | *Xanthoparmelia* | 21.55 | 13.64–32.74 | 1.27 | 0.41–2.35 |
| | *Myelochroa* | 17.55 | 10.00–26.23 | 3.36 | 1.45–6.35 |
| | *Punctelia* | 13.82 | 9.17–20.35 | 1.96 | 0.30–3.55 |
| | *Parmotrema* | 13.80 | 10.08–17.82 | 2.64 | 0.76–5.30 |
| | *Cetrelia* | 9.04 | 3.32–15.62 | 0.53 | 0.24–0.89 |
| | *Parmelina* | 8.45 | 2.24–19.49 | 0.68 | 0.22–1.25 |
| Neogene (Pliocene) 5.3–1.8 | *Canoparmelia crozalsiana*-clade | 3.91 | 1.68–7.09 | 3.36 | 1.77–5.36 |

Units. Ages: Ma. Rates: (s/s/y) $\times 10-9$. HPD: highest posterior density interval.
doi:10.1371/journal.pone.0028161.t002

**Table 3.** Estimated ages of the most recent common ancestor (MRCA) of the main clades obtained in the alternative BEAST analyses.

| | Only C1 + C2 | | Only C1 + C3 | | C1 + C2 + C3* | |
|---|---|---|---|---|---|---|
| **Node MRCA** | **Age** | **Height 95% HPD** | **Age** | **Height 95% HPD** | **Age** | **Height 95% HPD** |
| Lecanoromycetes | 282.74 | 243.97–323.49 | 298.72 | 264.94–327.29 | 297.23 | 263.04–325.17 |
| Ostropomycetidae | 229.14 | 189.55–268.83 | 249.46 | 216.10–280.61 | 250.70 | 216.87–282.32 |
| Lecanoromycetidae | 225.43 | 182.87–266.59 | 240.92 | 196.18–278.16 | 239.00 | 199.59–275.59 |
| Lecanorales | 125.74 | 95.55–157.78 | 134.20 | 103.23–107.77 | 142.62 | 110.27–172.06 |
| Parmeliaceae (*Protoparmelia* included) | 88.03 | 64.84–114.27 | 91.88 | 67.9–118.82 | 111.24 | 83.53–137.09 |
| Parmeliaceae (*Protoparmelia* excluded) | 58.49 | 46.05–72.06 | 61.08 | 49.41–74.53 | 74.10 | 59.11–89.74 |
| Parmelioid group | 48.13 | 39.33–62.63 | 51.06 | 40.71–61.64 | 59.66 | 49.94–71.67 |
| Split 1. *Xanthoparmelia – Parmotrema*-clade | 38.77 | 29.41–49.12 | 41.86 | 30.78–52.92 | 48.27 | 38.52–59.90 |
| Split 2. *Melanelixia - Melanohalea* | 32.68 | 22.59–43.43 | 35.78 | 25.65–46.41 | 41.28 | 31.45–52.33 |
| Split 3. *Parmelina – Myelochroa* | 29.35 | 17.59–41.00 | 31.50 | 20.84–40.76 | 37.03 | 24.50–48.71 |
| Split 4. *Remototrachyna - Bulbothrix* | 27.29 | 20.11–37.92 | 27.56 | 20.59–36.92 | 32.91 | 23.80–42.46 |
| Split 5. *Austroparmelina - Parmotrema* and *Flavoparmelia* | 28.45 | 20.81–36.38 | 30.72 | 22.37–39.69 | 35.60 | 26.90–45.42 |
| *Melanelixia* | 26.67 | 18.49–35.80 | 29.49 | 19.90–39.12 | 33.56 | 24.14–44.57 |
| Split 6. *Cetrariastrum- Hypotrachyna*-clade p.p. | 26.34 | 19.07–35.42 | 27.14 | 20.79–35.27 | 31.31 | 22.72–40.53 |
| Split 7. *Flavoparmelia - Parmotrema* | 31.38 | 23.46–39.95 | 26.33 | 18.33–33.90 | 30.52 | 23.48–39.49 |
| *Melanohalea* | 19.62 | 11.10–29.61 | 21.23 | 13.64–31.13 | 25.41 | 16.94–37.06 |
| *Parmelia* | 21.98 | 17.01–28.37 | 11.59 | 5.42–20.11 | 23.63 | 17.53–31.92 |
| *Remototrachyna* | 19.42 | 13.21–29.44 | 19.74 | 13.46–28.16 | 23.52 | 16.67–32.68 |
| *Flavoparmelia* | 18.39 | 12.21–25.41 | 19.77 | 12.41–26.68 | 22.79 | 16.25–30.77 |
| *Xanthoparmelia* | 17.76 | 10.00–27.89 | 19.75 | 12.88–29.62 | 21.99 | 13.03–34.65 |
| *Myelochroa* | 13.04 | 7.66–19.72 | 14.25 | 8.15–21.12 | 15.69 | 9.39–23.09 |
| *Punctelia* | 11.00 | 6.82–15.87 | 12.15 | 10.62–24.19 | 14.18 | 9.89–21.29 |
| *Parmotrema* | 11.44 | 7.57–16.04 | 12.45 | 7.55–16.79 | 14.38 | 10.26–19.46 |
| *Cetrelia* | 7.68 | 3.38–14.17 | 8.19 | 3.23–15.00 | 9.28 | 4.34–18.38 |
| *Parmelina* | 6.08 | 1.89–12.77 | 6.96 | 2.18–14.96 | 7.90 | 3.20–16.03 |
| *Canoparmelia crozalsiana*-clade | 3.25 | 1.42–5.45 | 3.31 | 1.47–5.94 | 3.95 | 1.69–7.38 |

Using the following calibration points: (1) C1 and C2 only; (2) C1 and C3 only; and (3) C1, C2 and C3* (with *Alectoria* fossil assigned to the alectorioid crown).Units: Ma. HPD: highest posterior density interval.

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fungi. In the case of lineages distributed at present in South America, Antarctica and Australia the estimated ages do not discard that vicariance could have resulted from the break-up of continents that had occurred 35–52 Ma.

For genera with more restricted distribution ranges and species groups occurring mainly in the Holarctic, additional phylogeographical data are necessary to test biogeographical hypotheses. This is the case e.g., for *Parmelina*, a genus that occurs in areas with a Mediterranean climate in the Northern Hemisphere [11]. Its separation from *Myelochroa*, a genus with center of distribution in eastern Asia [77] but extends further into temperate regions, was estimated as having happened in the late Eocene. Similarly the recently described genus *Remototrachyna*, a Southeast Asian element with only one pantropical species [19], diverged from its sister-lineage *Bulbothrix* also in the late Eocene.

Our analyses suggest a complex relationship of diversification events and palaeoclimatic conditions. The origin of Parmeliaceae was estimated in the late Cretaceous when the climate was warmer than today, and when subtropical to temperate fauna and flora extended well into polar latitudes [78–79]. The first radiation of the parmelioid group (60 Ma) occurred just before the Early Eocene Climatic Optimum (52 Ma) (Fig. 6) when temperature and atmospheric $CO_2$ reached maximum levels [80]. During this time period, the general climate was warm and humid, associated with tectonic changes and volcanism [81–82]. Most of the parmelioid lineages, however originated during the Eocene cooling and Oligocene glaciation (Fig. 6). During the Eocene–Oligocene transition (33.5 Ma) a profound global climate shift took place changing the Cretaceous/early Palaeogene "Green House" conditions to "Ice House" conditions, with the growth of Antarctic ice sheets to approximately their modern size, and the appearance of Northern Hemisphere glacial ice [80]. The radiation of the genera took place at different times from the early Oligocene to the Pliocene. *Melanelixia* started to radiate during the early Oligocene. During this time, the general climate was characterized for cold conditions of the Oligocene glaciation (B, Fig. 6). However *Melanohalea*, *Parmelia*, *Remototrachyna*, *Flavoparmelia*, *Xanthoparmelia* and *Myelochroa* started to radiate between the Late Oligocene Warming. and the Mid-Miocene Climatic Optimum (C–D, Fig. 6). The radiation of other genera (*Punctelia*, *Parmotrema*, *Cetrelia*, *Parmelina*, *Canoparmelia crozalsiana*-clade) is estimated as having occurred at the transition from the middle Miocene to the Early Pliocene, before the climate became cooler, drier and seasonal at the end of Pliocene [80].

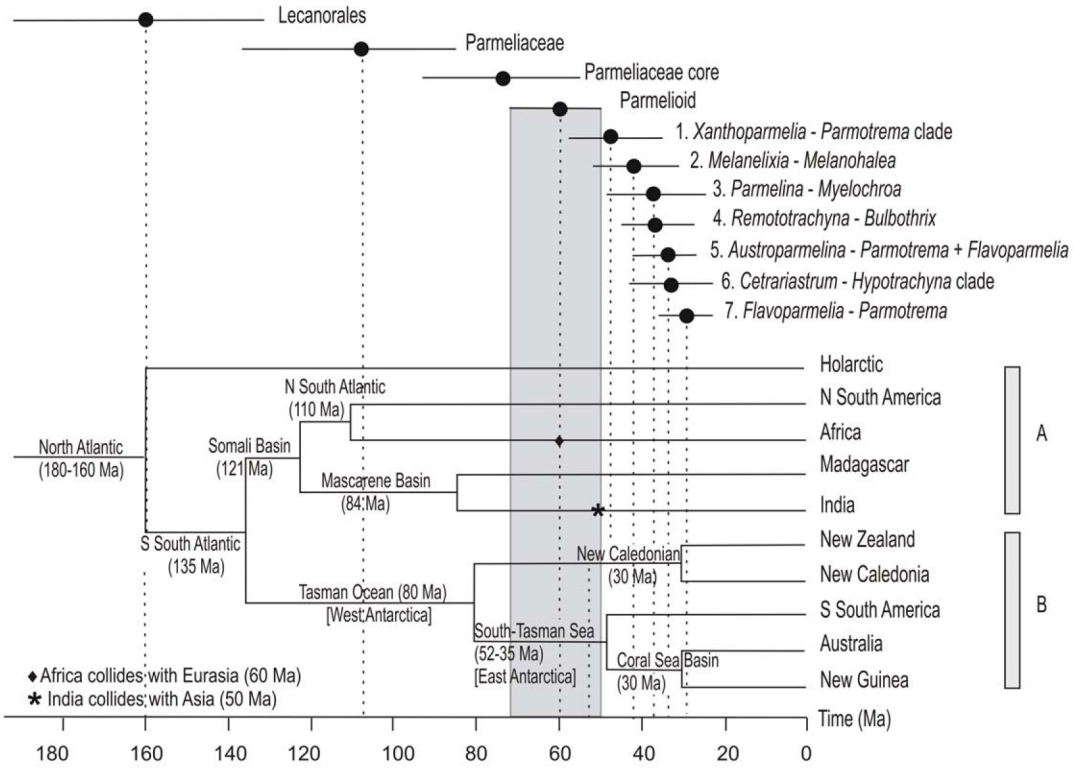


**Figure 5. Comparison of divergence ages of parmelioid clades and the separation of the Southern Hemisphere landmasses.** Dots represent node ages and bars cover the 95%HPD. The geological area cladogram representing the relationships among the Southern Hemisphere landmasses is based on Sanmartín & Ronquist [106].
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## Conclusions

Using three calibration points (one at the split of Lecanoromycetes and Eurotiomycetes, and the ages of two fossil lichens) we obtained the first dated phylogeny of parmelioid lichens and estimated the ages of divergence of the well-resolved lineages and main genera. The radiation of the Parmelioid occurred near the Cretaceous-Tertiary (K/T) boundary, before the climatic optima. These age estimates indicate that long-distance dispersal has played a major role in shaping the current distribution of the Southern Hemisphere parmelioid lichens and that continental drift of Gondwana landmasses and vicariance cannot explain the Africa-Australia disjunct patterns. The major genera originated during Eocene and Oligocene, and radiated during cooling periods at different times from the late Oligocene to early Pliocene.

## Materials and Methods

### Taxon sampling, sequence alignment and selection of substitution model

A dataset of 225 specimens of Lecanoromycetes with complete sequences of nuLSU rDNA, mtSSU rDNA and the protein coding *RPB1* gene, generated in previous studies [18,83–84], was compiled for this study. GenBank accession numbers are listed in Table S1. The parmelioid lineages (the main focus of this work) were represented by 96 OTUs including 24 genera. The major lineages of Lecanoromycetes [85–86] and outgroups (Chaethothyriomycetidae and Dothideomycetes), represented by 129 OTUs were included in the analyses to prevent that uneven sampling across the tree could distort the apparent trend in speciation through time [87]. The sequences of each locus were aligned separately using Muscle 3.6 [88] and the ambiguous positions removed using Gblocks with default settings [89–90]. The general time reversible model including estimation of invariant sites (GTR+I+G) was selected by jModelTest v 0.1.1 [91–92] as the most appropriate nucleotide substitution model for the three separated loci.

### Calibration of nodes and dating analysis

The divergence time analyses were performed using BEAST v.1.6.1 [93]. For the dating analysis it is recommended to use a user starting tree instead of the random starting tree built by BEAST. The latter is very likely to violate the temporal and/or topological constraints specified to calibrate divergence times, and cause an error when attempting to initiate the MCMC. For building this tree we checked the phylogenetic signal of our matrix running preliminary ML and Bayesian analyses using Garli 0.96 [94] and MrBayes 3.1.1 [95]. ML analyses were carried out with the default settings and Bayesian analyses were performed assuming a GTR+I+G model, run for 5 million of generations with 4 chains and every $100^{th}$ tree sampled. The first 5000 generations were burned in and a majority rule consensus tree was calculated with the sumt option. The topologies generated separately for each locus by ML and Bayesian analyses were congruent with the topology of the three loci concatenated, and with the general phylogeny obtained by Crespo *et al.* [18].

Three points of calibration were used for this study. The principal calibration point (C1) was the divergence time of 280–330 Ma for the stem of Lecanoromycetes following [26]. In addition we used the ages of two fossil lichens: the diversification node (C2) of *Parmelia* was calibrated with fossils from the

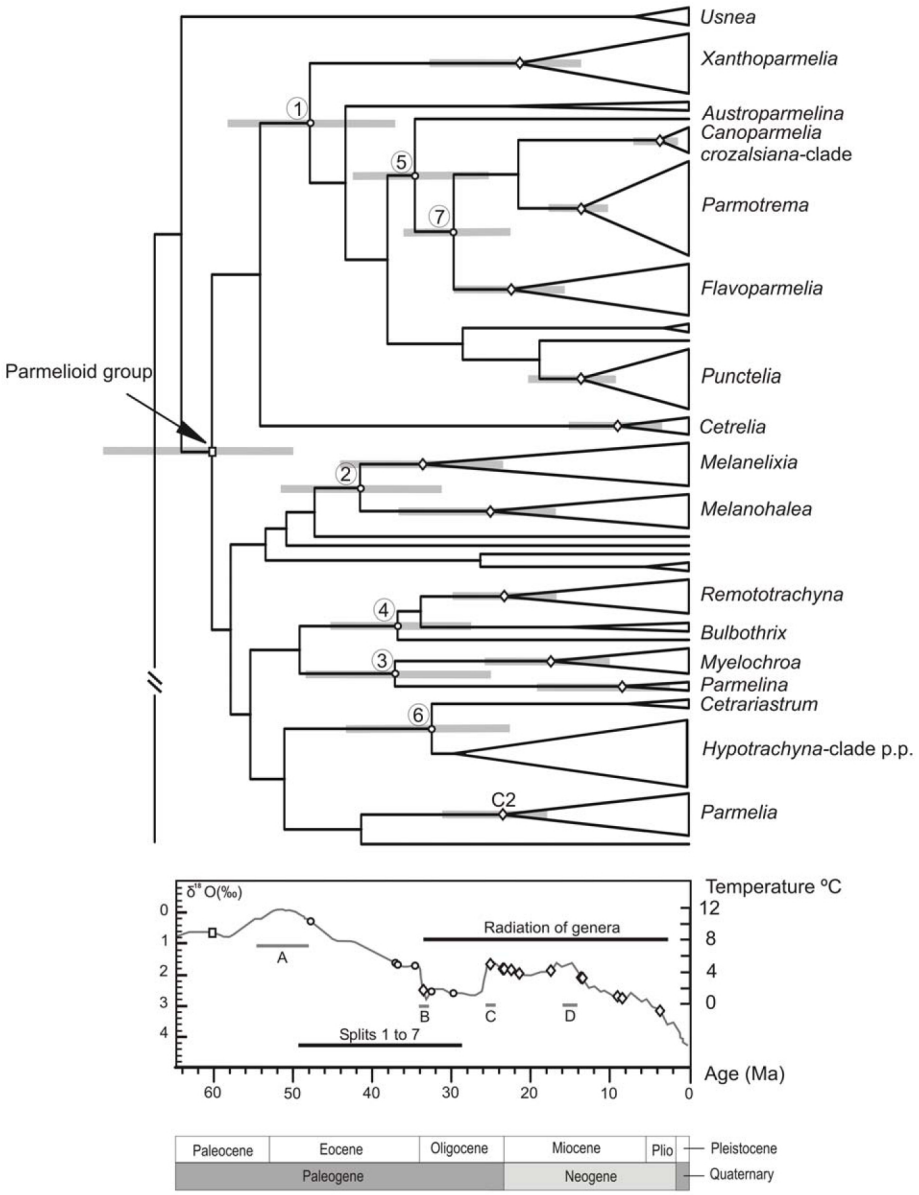


**Figure 6. Chronogram of parmelioid genera during Cenozoic time and its relationship to global temperature changes.** The global temperature changes obtained from the deep-sea oxygen and carbon isotype proxies after Zachos *et al* [107].The major diversification events of parmelioid lichens are mapped onto the temperature curve: the square represents the parmelioid most recent common ancestor (MCRA), circles indicate splits of major lineages (numbers in the tree refer to those in Table 2), and diamonds the radiation of genera. Climatic events: A. Early Eocene Climatic Optimum. B. Oi-1 Glaciation. C. Late Oligocene Warming. D. Mid-Miocene Climatic Optimum.
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Dominican amber (*Parmelia ambra*, 15–45 Ma, [50]), and the crown node (C3) of *Alectoria* with a fossil from the Baltic amber (35–40 Ma, [49]). The assignation of fossils to extant groups is a crucial matter in dating analyses, and in the case of the lichens the fossil record is so sparse that this becomes particularly important. *Parmelia ambra* is a fossil from the Dominican amber resembling *Parmelia saxatilis* and similar species [50]. It presents unclear terminal pseudocyphellae, elongate isidia, plane to concave upper surface, and simple to dichotomously branched rhizines. All these features are characteristic of the *Parmelia s. s.* clade, and thus the fossil could also be related to the phylogenetically close genus *Relicina* (sister group of the *Parmelia* s.s.), the '*Parmelia signifera*' group, or to the morphologically close *Nipponoparmelia* [18]. *Relicina* was discarded because *P. ambra* does not have cilia, a feature present on the *Relicina* species. The species of the '*Parmelia signifera*' group have subsquarrose rhizines different from the simple to dichotomously branched rhizines of the fossil. On the other hand, *Nipponoparmelia* presents lobes rolled upwards [96] different from the flat lobes of the fossil. Moreover, only *N. isidioclada* has isidia but the rhizines are much branched, not simple or bifurcate, than those of the fossil. Thus, the *Parmelia ambra* fossil was used to calibrate the *Parmelia* s. s. crown node in the starting tree.

The *Alectoria* fossil from the Baltic amber [49] is morphologically related to the alectorioid clade (*Bryoria*, *Pseudophebe* and *Alectoria*; [35]). The fossil has abundant apothecia, a character that it shares with *Alectoria*, while the related *Bryoria* and *Pseudephebe* genera rarely have apothecia. Thus this fossil was used to constrain the crown node of *Alectoria*. Nevertheless, due to the inevitable uncertainty in

placement of fossil taxa we assessed the impact of individual fossil calibration on divergence time estimates using alternative analysis: 1) using a single fossil for calibration, either *Parmelia* (C2 node) or *Alectoria* (C3 node); and 2) using the *Alectoria* fossil to calibrate the whole alectorioid clade, including *Alectoria*, *Bryoria* and *Pseudephebe* (C3*).

The divergence time analyses were performed using BEAST v.1.6.1 [93]. We used as starting tree the ML tree obtained with Garli 0.96 [94], made ultrametric using nonparametric rate smoothing (NPRS) implemented in TreeEdit v.10a10 [97] with the divergence between Lecanoromycetes and Chaethothyriomycetidae set at 305 Ma. We constrained the position of Chaethothyriomycetidae as sister clade of Lecanoromycetes based on Schoch *et al.* [98]. Previously to the analysis, we test that this constraint is not significantly worse than the unconstrained topology, using the Shimodaira-Hasegawa test (SH) [99] and Expected Likelihood Weights test (ELW) [100]. Both tests were run on Tree-Puzzle 5.2 [101].

The final dating analysis was performed with a partitioned BEAST analysis with unlinked substitutions models (GTR+I+G) across the loci, a Birth-Death process tree prior, and a relaxed clock model (uncorrelated lognormal) for each partition. Calibration points were defined as prior distributions: 1) the split of Lecanoromycetes and Chaethothyriomycetidae (C1) was calibrated with a uniform distribution (280–330 Ma). 2) The calibrations points with fossils were considered as minimal ages and calibrated with a lognormal distribution [102]. The *Parmelia* crown node (C2) at log-normal mean = 2.77, offset = 14, lognormal standard deviation = 0.5. The *Alectoria* crown node (C3) at lognormal mean = 3.61, offset = 34, lognormal standard deviation = 0.75. The final analysis was run for 10 million generations, with parameter values sampled every 1000 generation. We checked the stationary plateau with Tracer v. 1.4.1 [103]. We discarded 10% of the initial trees as burn in and the consensus tree was calculated using Tree Annotator v 1.6.1 [96]. The results were visualized with FigTree v. 1.3.1 [104]. The substitution rates for each locus were obtained running independent BEAST analyses for each dataset using the same parameters as in the partitioned analysis. Ages and rates were estimated for all the nodes with more than 0.95 of posterior probability both in the BEAST runs and in the previous Bayesian analysis.

## Supporting Information

**Table S1 Specimens used in this study with GenBank accession numbers.**
(DOC)

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## Author Contributions

Performed the experiments: GAdP PKD. Analyzed the data: GAdP PC PKD. Contributed reagents/materials/analysis tools: GAdP PC PKD HTL AC. Wrote the paper: GAdP PC PKD HTL AC. Contributed to analyses and discussion: GAdP PC PKD HTL AC.

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## Supporting information

### Table S1. Specimens used in this study with GenBank accession numbers.

| | GenBank accession no. | | |
|---|---|---|---|
| Species | nuLSU | mtSSU | *rpb1* |
| *Acarospora schleicheri* | AY853353 | AY853305 | DQ915591 |
| *Ainoa geochroa* | DQ871006 | DQ871015 | DQ870927 |
| *Ainoa mooreana* | AY212828 | AY212850 | DQ870928 |
| *Alectoria nigricans* | DQ923649 | DQ923620 | DQ923676 |
| *Alectoria ochroleuca* | DQ899288 | DQ899289 | DQ923677 |
| *Alectoria sarmentosa* | DQ899290 | DQ899291 | DQ923678 |
| *Allantoparmelia alpicola* | DQ923650 | DQ923621 | DQ923679 |
| *Arctomia delicatula* | AY853355 | AY853307 | DQ870929 |
| *Arctomia teretiuscula* | DQ007346 | DQ007349 | DQ870930 |
| *Arthrorhaphis citrinella* | AY853356 | AY853308 | DQ915592 |
| *Aspicilia caesiocinerea* | DQ780303 | DQ780271 | DQ870931 |
| *Aspicilia cinerea* | DQ780304 | DQ780272 | DQ870932 |
| *Aspicilia izcoana* | AY853359 | AY853311 | DQ870934 |
| *Austroparmelina pruinata* | EF042914 | EF025481 | GU994680 |
| *Arctocetraria andrejevii* | DQ923652 | DQ923623 | DQ923680 |
| *Arctoparmelia centrifuga* | AY578917 | AF351156 | EF092099 |
| *Baeomyces placophyllus* | AY300658 | AF356878 | DQ870936 |
| *Baeomyces rufus* | DQ871008 | DQ871016 | DQ870937 |
| *Brodoa atrofusca* | AY607824 | AY643090 | EF092100 |
| *Brodoa intestiniformis* | DQ923653 | DQ923624 | DQ923681 |
| *Brodoa oroarctica* | DQ923654 | DQ923625 | DQ923682 |
| *Bryoria capillaris* | DQ923655 | DQ923626 | DQ923683 |
| *Bryoria fremontii* | DQ923656 | DQ923627 | DQ923684 |
| *Bryoria fuscescens* | EF042912 | AF351158 | EF092101 |
| *Bulbothrix coronata* | EU562671 | DQ287789 | GU994681 |
| *Bulbothrix decurtata* | EU562672 | DQ287790 | GU994682 |
| *Bulbothrix meizospora* | AY607780 | AY611127 | EF092102 |
| *Caloplaca flavorubescens* | AY300831 | AY143403 | DQ915593 |
| *Candelaria concolor* | EF436461 | EF436460 | EF436461 |
| *Candelariella aurella* | AY853361 | AY853313 | DQ915594 |
| *Cladonia rangiferina* | AY300832 | AY300881 | DQ915595 |
| *Cladosporium* sp. | AY016367 | AY571386 | DQ870938 |
| *Canoparmelia carneopruinata* | EF042913 | EF025480 | GU994684 |
| *Canoparmelia caroliniana* | AY584634 | AY584613 | DQ782817 |
| *Canoparmelia crozalsiana* | AY584831 | AY586594 | EF092104 |
| *Canoparmelia inhaminensis* | GU994586 | GU994633 | GU994686 |
| *Canoparmelia schelpei* | GU994588 | GU994634 | GU994688 |
| *Canoparmelia texana* | EF042915 | EF025482 | EF092105 |
| *Cetraria islandica 1* | DQ912334 | AY340486 | DQ912356 |
| *Cetraria islandica 2* | AY340539 | DQ912277 | DQ923685 |
| *Cetrariastrum andense* | GQ919245 | GQ919217 | GU994690 |
| *Cetrariastrum dubitans* | GQ919246 | GQ919217 | GU994691 |
| *Cetrariella delisei* | DQ923657 | DQ923628 | DQ923686. |
| *Cetrelia cetrarioides* | GU994591 | GU994636 | GU994692 |
| *Cetrelia olivetorum* | DQ923659 | DQ923630 | GU994693 |
| *Cetrelia pseudolivetorum* | GU994594 | GU994639 | GU994694 |
| *Coccotrema cucurbitula* | AF274092 | AF329161 | DQ870939 |
| *Coccotrema pocillarium* | AF274093 | AF329166 | DQ870940 |
| *Cornicularia normoerica* | DQ923661 | DQ923632 | DQ923687 |
| *Dactylina arctica* | DQ986802 | DQ986786 | DQ986859 |
| *Diploschistes cinereocaesius* | AY300835 | AY300885 | DQ870941 |
| *Diploschistes scruposus* | AF279389 | AY584692 | DQ870943 |
| *Emodomelanelia masonii* | GU994595 | GU994640 | GU994695 |

| | GenBank accession no. | | |
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| Species | nuLSU | mtSSU | *rpb1* |
| *Evernia prunastri 1* | AF107562 | DQ923634 | EF105428 |
| *Evernia prunastri 2* | AF113745 | AF351162 | DQ870944 |
| *Everniastrum nepalense* | AY607783 | AY611129 | EF092106 |
| *Everniopsis trulla* | EF108290 | EF108289 | EF105429 |
| *Flavocetraria nivalis 1* | DQ883795 | DQ923635 | DQ883738 |
| *Flavocetraria nivalis 2* | AY533003 | DQ912278 | DQ923688 |
| *Flavoparmelia caperata 1* | AY584639 | AY584617 | EF092107 |
| *Flavoparmelia caperata 2* | AY578922 | AF351163 | DQ870945 |
| *Flavoparmelia citrinescens* | GU994596 | GU994641 | GU994696 |
| *Flavoparmelia marchantii* | GU994598 | GU994642 | GU994698 |
| *Flavoparmelia soredians* | AY584835 | AY586586 | EF092108 |
| *Flavoparmelia springtonensis* | EF042916 | EF025483 | EF092109 |
| *Flavoparmelia subambigua* | GU994599 | GU994643 | GU994699 |
| *Flavopunctelia flaventior 1* | DQ912335 | DQ912279 | EF092110 |
| *Flavopunctelia flaventior 2* | AY578923 | AY586587 | DQ912357 |
| *Graphis scripta* | AY853370 | AY853322 | DQ870947 |
| *Gregorella humida* | AY853378 | AY853329 | DQ870946 |
| *Hypocenomyce scalaris* | AY853373 | AY853325 | DQ915596 |
| *Hypogymnia vittata* | DQ900637 | DQ900629 | DQ923689 |
| *Hypotrachyna polydactyla* | GQ919258 | GQ919231 | GU994703 |
| *Hypotrachyna revoluta* | AY607787 | AF351166 | EF092112 |
| *Hypotrachyna sinuosa* | AY607788 | AY611133 | EF092113 |
| *Imshaugia aleurites 1* | DQ986753 | DQ986864 | EF092114 |
| *Imshaugia aleurites 2* | AY607840 | AF351167 | DQ986825 |
| *Lecanora hybocarpa* | EF105421 | EF105417 | DQ870949 |
| *Lecanora paramerae* | EF105422 | EF105418 | DQ870950 |
| *Lecanora sulphurea* | EF105423 | EF105419 | DQ870951 |
| *Lepraria usnica* | AY300843 | AY300894 | DQ870952 |
| *Lethariella cashmeriana* | DQ923665 | DQ923637 | DQ923690 |
| *Lobaria pulmonaria* | AF183934 | AF069541 | DQ915597 |
| *Lobothallia radiosa* | DQ780306 | DQ780274 | DQ870954 |
| *Loxospora ochrophaea* | DQ871009 | DQ871017 | DQ870953 |
| *Masonhalea richardsonii* | DQ973031 | DQ972979 | DQ973054 |
| *Melanelia disjuncta* | AJ421431 | DQ923638 | DQ923691 |
| *Melanelia hepatizon* | DQ923667 | DQ923639 | DQ923692. |
| *Melanelia sorediata* | GU994604 | GU994645 | GU994706 |
| *Melanelia sorediella* | GU994606 | GU994646 | GU994707 |
| *Melanelia stygia* | AJ421434 | DQ923640 | DQ923693 |
| *Melanelixia fuliginosa 1* | AJ421428 | DQ986787 | EF092116 |
| *Melanelixia fuliginosa 2* | AJ421435 | AY611179 | DQ986860 |
| *Melanelixia glabra* | AJ421427 | GU994651 | EF092118 |
| *Melanelixia subargentifera* | AJ421429 | AY611155 | EF092119 |
| *Melanelixia subaurifera* | AJ421432 | AY611174 | EF092120 |
| *Melanelixia subglabra* | GU994610 | GU994654 | GU994711 |
| *Melanohalea elegantula* | AJ421437 | AY611151 | EF092122 |
| *Melanohalea exasperata 1* | AJ421438 | AY611140 | EF092124 |
| *Melanohalea exasperata 2* | AY607795 | AY611138 | EF092123 |
| *Melanohalea olivacea* | AY607803 | AY611148 | EF092125 |
| *Melanohalea subelegantula* | AY607829 | AY611171 | EF092126 |
| *Menegazzia terebrata* | AY584637 | DQ899305 | DQ923694 |
| *Myelochroa aurulenta 1* | DQ973025 | EF025484 | DQ973049 |
| *Myelochroa aurulenta 2* | EF042917 | DQ972972 | EF092127 |
| *Myelochroa irrugans* | AY607815 | AY611160 | EF092128 |
| *Myelochroa metarevoluta* | AY607814 | AY611159 | EF092129 |
| *Myriangium duriaei* | AY016365 | AY571389 | DQ870956 |
| *Nesolechia oxyspora* | DQ923669 | DQ923642 | GU994712 |
| *Ochrolechia androgyna* | AY300846 | AY300897 | DQ870957 |
| *Ochrolechia oregonensis* | DQ780308 | DQ780276 | DQ870958 |

| | GenBank accession no. | | |
|---|---|---|---|
| Species | nuLSU | mtSSU | *rpb1* |
| *Ochrolechia parella* | AF274097 | AF320173 | DQ870959 |
| *Ochrolechia turneri* | AY568002 | AY567982 | DQ870961 |
| *Omphalodium pisacomense* | GU994617 | GU994663 | GU994715 |
| *Orceolina antarctica* | AY212115 | AF274852 | DQ870962 |
| *Orceolina kerguelensis* | AY212830 | AF381561 | DQ870963 |
| *Pannoparmelia angustata* | AY785265 | AF351170 | EF092131 |
| *Parmelia discordans* | EF042918 | DQ287841 | EF092132 |
| *Parmelia saxatilis* | AY300849 | AY340514 | DQ923695 |
| *Parmelia serrana* | AY578948 | AY582319 | EF092133 |
| *Parmelia squarrosa* | AY607816 | AY611162 | EF092134 |
| *Parmelia sulcata* | AY578949 | AY582320 | EF092135 |
| *Parmelia sulcata 2* | GU994669 | GU994621 | GU994720 |
| *Parmelina carporrhizans* | AY607818 | AY611164 | EF092136 |
| *Parmelina tiliacea* | AY578950 | AF351173 | EF092137 |
| *Parmelinopsis afrorevoluta* | GQ919259 | GQ919233 | GU994722 |
| *Parmelinopsis horrescens* | AY578951 | AY582321 | EF092138 |
| *Parmelinopsis minarum* | AY578952 | AY582322 | EF092139 |
| *Parmelinopsis neodamaziana* | AY607820 | AY611166 | EF092140 |
| *Parmelinopsis subfatiscens* | AY607821 | AF351174 | EF092141 |
| *Parmeliopsis hyperopta* | AY607823 | AY611167 | EF092142 |
| *Parmotrema cetratum* | AY584847 | AY648935 | EF092143 |
| *Parmotrema crinitum* | AY584837 | EU562699 | GU994723 |
| *Parmotrema fistulatum* | AY578920 | EU562700 | GU994724 |
| *Parmotrema haitiense* | AY578918 | AY582295 | EF092144 |
| *Parmotrema hypoleucinum* | AY584839 | AY586590 | GU994725 |
| *Parmotrema perforatum* | AY584840 | AY586591 | EF092145 |
| *Parmotrema perlatum* | AY584838 | AY586580 | EF092146 |
| *Parmotrema pilosum* | AY578919 | EU562701 | GU994728 |
| *Parmotrema reticulatum 1* | DQ912339 | DQ912283 | DQ912361 |
| *Parmotrema reticulatum 2* | AY584850 | AY648933 | GU994729 |
| *Parmotrema subtinctorium* | AY584830 | AY586582 | GU994730 |
| *Parmotrema tinctorum* | AY584635 | AY584627 | DQ912362 |
| *Peltigera aphthosa* | AF286759 | AY340515 | DQ915598 |
| *Pertusaria albescens* | AF329176 | AF329175 | DQ870964 |
| *Pertusaria amara* | AF274101 | AY300900 | DQ870965 |
| *Pertusaria coccodes* | AF279295 | AY567984 | DQ870966 |
| *Pertusaria corallophora* | DQ780316 | DQ780285 | DQ870969 |
| *Pertusaria coronata* | AY300851 | AY300902 | DQ879068 |
| *Pertusaria gibberosa* | DQ780322 | DQ780289 | DQ870970 |
| *Pertusaria hemisphaerica* | AF381556 | AF381563 | DQ902341 |
| *Pertusaria lactea* | AF381557 | AF381564 | DQ870971 |
| *Pertusaria lecanina* | AF279296 | AY567991 | DQ870972 |
| *Pertusaria leioplaca* | AY300852 | AY300903 | DQ870973 |
| *Pertusaria mammosa* | AY212831 | AY212854 | DQ870974 |
| *Pertusaria mesotrapa* | DQ780325 | DQ780292 | DQ870975 |
| *Pertusaria ophthalmiza* | AY568006 | AY567993 | DQ870976 |
| *Pertusaria panyrga* | DQ780327 | AY567994 | DQ870977 |
| *Pertusaria pertusa* | AF279300 | AF381565 | DQ870978 |
| *Pertusaria plittiana* | DQ780328 | DQ780294 | DQ870979 |
| *Pertusaria scaberula* | AF274099 | AF431959 | DQ870980 |
| *Pertusaria subventosa* | AY300854 | AY300905 | DQ870981 |
| *Pertusaria velata* | AY300855 | AY300906 | DQ870982 |
| *Placopsis cribellans* | DQ871010 | DQ871018 | DQ870983 |
| *Placopsis gelida* | AY212836 | AY212859 | DQ870984 |
| *Placopsis santessonii* | AY212845 | AY212867 | DQ870986 |
| *Placynthiella icmalea* | AY212846 | AY212870 | DQ870985 |
| *Platismatia glauca 1* | DQ973032 | AY756404 | DQ912363 |
| *Platismatia glauca 2* | DQ912340 | DQ972980 | DQ973055 |

| | GenBank accession no. | | |
|---|---|---|---|
| Species | nuLSU | mtSSU | *rpb1* |
| *Platismatia norvegica* | DQ923671 | DQ923644 | DQ923696 |
| *Pleurosticta acetabulum* | AY578953 | AY582323 | EF092147 |
| *Protoparmelia badia 1* | DQ431917 | EF105420 | EF105434 |
| *Protoparmelia badia 2* | DQ431916 | DQ899311 | DQ870987 |
| *Protothelenella corrosa* | AY607734 | AY607746 | DQ870988 |
| *Protothelenella sphinctrinoidella* | AY607735 | AY607747 | DQ870989 |
| *Protousnea magellanica* | DQ985193 | DQ985194 | DQ985195 |
| *Pseudephebe pubescens* | AY607839 | AF351180 | EF092148 |
| *Pseudevernia consocians* | DQ986754 | DQ986868 | DQ986826 |
| *Pseudevernia furfuracea* | AY607826 | AF351181 | EF105435 |
| *Punctelia borreri* | AY578954 | AY582324 | EF092149 |
| *Punctelia jeckeri* | AY613427 | GU994625 | GU994731 |
| *Punctelia pseudocoralloidea* | AY584843 | AY586595 | EF092150 |
| *Punctelia reddenda* | GU994627 | AY613430 | GU994732 |
| *Punctelia rudecta 1* | AY584636 | AY584630 | EF092151 |
| *Punctelia rudecta 2* | AY584845 | GU994672 | DQ912365 |
| *Punctelia* sp. | GU994628 | GU994673 | GU994733 |
| *Punctelia subflava* | AY584846 | EU562704 | GU994734 |
| *Relicina subnigra* | AY785267 | AY785281 | EF092152 |
| *Remototrachyna ciliata* | AY785266 | AY785280 | EF092111 |
| *Remototrachyna costaricensis* | AY785262 | AY785276 | GU994736 |
| *Remototrachyna incognita* | EU562687 | DQ287815 | GU994737 |
| *Remototrachyna infirma* | AY785264 | AY785278 | GU994738 |
| *Remototrachyna scytophylla* | EU562694 | DQ287836 | GU994739 |
| *Rhizocarpon sphaerosporum* | AY853390 | AY853340 | DQ870991 |
| *Rimularia psephota* | DQ871012 | DQ871019 | DQ870992 |
| *Schaereria corticola* | AY300859 | AY300909 | DQ870993 |
| *Sporastatia testudinea* | AY640969 | AY584725 | DQ870994 |
| *Staurothele fissa* | DQ329028 | DQ329003 | DQ870995 |
| *Staurothele rufa* | DQ329029 | DQ329004 | DQ70996 |
| *Thamnolia vermicularis* | AY853395 | AY853345 | DQ915599 |
| *Thelotrema subtile* | DQ871013 | DQ871020 | DQ870997 |
| *Thelotrema suecicum* | AY300867 | AY300917 | DQ870998 |
| *Trapelia chiodectonoides* | AY212847 | AY2128873 | DQ870999 |
| *Trapelia placodioides* | AF274103 | AF431962 | DQ366259 |
| *Trapeliopsis flexuosa* | AF274118 | AY212875 | DQ871000 |
| *Trapeliopsis granulosa* | AF274119 | AF381561 | DQ871001 |
| *Trapeliopsis percrenata* | AF279302 | AY212876 | EF158853 |
| *Tuckermannopsis chlorophylla* | DQ923674 | DQ923647 | DQ923697 |
| *Umbilicaria crustulosa* | AY300869 | AY300919 | DQ871002 |
| *Umbilicaria decussata* | AY603113 | DQ571021 | DQ871003 |
| *Umbilicaria hyperborea* | AY853399 | AY853349 | DQ915600 |
| *Usnea antarctica 1* | DQ883692 | DQ990920 | DQ883721 |
| *Usnea antarctica 2* | DQ899309 | EF116571 | EF193050 |
| *Usnea trachycarpa* | EF116570 | EF116572 | EF193058 |
| *Vulpicida pinastri 1* | DQ912341 | DQ923648 | DQ912366 |
| *Vulpicida pinastri 2* | DQ923675 | DQ912285 | DQ923698 |
| *Wawea fruticulosa* | DQ007347 | DQ871023 | DQ871005 |
| *Xanthoparmelia azaniensis* | EF042910 | EF025478 | EF092098 |
| *Xanthoparmelia conspersa 1* | AY584641 | DQ899314 | EF092155 |
| *Xanthoparmelia conspersa 2* | DQ899313 | AY584633 | DQ912367 |
| *Xanthoparmelia exornata* | EF108318 | EF025485 | EF092130 |
| *Xanthoparmelia hottentotta* | EF042919 | EF025486 | EF092153 |
| *Xanthoparmelia mougeotii* | AY578966 | AY582336 | EF092156 |
| *Xanthoparmelia saxeti* | AY578926 | AY582299 | EF092115 |
| *Xanthoparmelia semiviridis* | AY578921 | AF351160 | EF092157 |

# La dispersión transoceánica y posterior diversificación en cada continente modeló la diversidad en el grupo *Xanthoparmelia pulla* (Ascomycota)

Guillermo Amo de Paz, Paloma Cubas, Ana Crespo, John A. Elix & H. Thorsten Lumbsch

PlosOne 7 (6): e39683 (2012)

## Resumen

Según el concepto fenotípico de especie, muchos hongos liquenizados poseen amplias distribuciones, abarcando varios continentes de ambos Hemisferios. Los datos moleculares están revolucionando la delimitación de especie en estos hongos y han demostrado que subestimábamos la diversidad de estos organismos. El objetivo de este estudio es explorar la biogeografía y los patrones evolutivos de los líquenes del grupo '*Xanthoparmelia pulla'*, un conjunto de especies ampliamente distribuido de uno de los géneros de macrolíquenes más diverso. Usando una filogenia datada basada en secuencias de nuITS y nuLSU de ADN ribosómico hemos realizado una reconstrucción del área ancestral del grupo y datado los tiempos de divergencia de los principales linajes para entender los procesos de dispersión y explicar su distribución actual. Los puntos de calibración de la filogenia fueron la edad de radiación inferida para los líquenes parmelioides y la edad de un fósil del género *Parmelia*. Los resultados muestran que, tal cuál están delimitadas las especies siguiendo el concepto fenotípico, muchas de las que presentan una amplia distribución en varios continentes son polifiléticas. Sin embargo, la filogenia muestra cinco linajes principales que se correlacionan con la distribución geográfica de los especímenes y con las rutas biosintéticas de los metabolitos secundarios. Según los resultados del análisis de estimación de los tiempos de divergencia y del análisis de reconstrucción del área ancestral, Sudáfrica es el lugar donde con una mayor probabilidad el grupo comenzó su diversificación durante el Mioceno. La edad de radiación del grupo coincide con el aumento de aridez en la Región del Cabo debida a las corrientes oceánicas. Este aumento de la aridez en el Mioceno se relaciona con la actual riqueza de flora adaptada a condiciones xéricas. En el momento presente del planeta esta es la región con más diversidad genética, morfológica y química del grupo '*X. pulla*'. Desde este centro de radiación, diferentes linajes migraron vía dispersión a larga distancia a otras áreas, donde sufrieron radiaciones secundarias. También podemos estimar que desde la radiación secundaria producida en Australia se ha dispersado un linaje a Sudamérica por dispersión a larga distancia y allí ha comenzado una nueva radiación.

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# Transoceanic Dispersal and Subsequent Diversification on Separate Continents Shaped Diversity of the *Xanthoparmelia pulla* Group (Ascomycota)

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## Abstract

In traditional morphology-based concepts many species of lichenized fungi have world-wide distributions. Molecular data have revolutionized the species delimitation in lichens and have demonstrated that we underestimated the diversity of these organisms. The aim of this study is to explore the phylogeography and the evolutionary patterns of the *Xanthoparmelia pulla* group, a widespread group of one of largest genera of macrolichens. We used a dated phylogeny based on nuITS and nuLSU rDNA sequences and performed an ancestral range reconstruction to understand the processes and explain their current distribution, dating the divergence of the major lineages in the group. An inferred age of radiation of parmelioid lichens and the age of a *Parmelia* fossil were used as the calibration points for the phylogeny. The results show that many species of the *X. pulla* group as currently delimited are polyphyletic and five major lineages correlate with their geographical distribution and the biosynthetic pathways of secondary metabolites. South Africa is the area where the *X. pulla* group radiated during the Miocene times, and currently is the region with the highest genetic, morphological and chemical diversity. From this center of radiation the different lineages migrated by long-distance dispersal to others areas, where secondary radiations developed. The ancestral range reconstruction also detected that a secondary lineage migrated from Australia to South America via long-distance dispersal and subsequent continental radiation.

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## Introduction

Methods for delimiting species, the fundamental taxonomic unit, have always fascinated evolutionary biologists [1–3]. Understanding the circumscription of species is important for biological and ecological studies and for conservation issues. However, the main challenge is to recognize species in organisms with relatively simple morphologies. In lichenized fungi traditional species circumscriptions are based on phenotypic characters, such as thallus and ascomatal morphology or chemical characters. However, there is a growing body of evidence from molecular studies that the traditional morphology-based species circumscriptions are insufficient to represent the diversity in lichenized ascomycetes [4–26]. A number of DNA sequence-based phylogenetic studies revealed the presence of distinct lineages within currently delimited species. Subsequent, detailed studies often revealed previously overlooked morphological subtleties or chemical differences among those clades and authors often refer to these as "semi-cryptic" species [8].

On a par with the phenotypic-based species circumscription, researchers often accepted wide distribution ranges for species occurring on different continents. This was at least partially due to a common belief by lichenologists in the "everything is everywhere" hypothesis [27,28] applied to fungi, as discussed elsewhere [8,29]. In several cases molecular data assisted in a better understanding of the biogeography of lichen-forming fungi where taxa were shown to represent different species on different continents, e.g. the *Leptogium furfuraceum* aggr. on different continents [18], *Melanelixia glabra* s. lat. in Europe and North America [14], *Parmelina quercina* s. lat. on different continents [4], *Physcia aipolia* aggr. in Europe and Australia [9], *Xanthoparmelia* spp. in North America and Australia [15,16]. In the *Leptogium furfuraceum* aggr. complex sister-group relationships were found between populations from the same hemispheres which were incongruent with previous classifications based on morphological differences [18], and the dated phylogeny indicated that the species had migrated via transoceanic dispersal to different continents.

Here we report another case of a group of lichenized fungi where transoceanic dispersal to different continents is correlated with the phylogenetic lineages. The group studied here is the *Xanthoparmelia pulla* group which belongs to the family Parmeliaceae. This family represents one of the largest families of lichenized fungi [30,31]. The main clade of the family is the parmelioid clade with almost 2000 species [32] currently classified in 27 genera, with *Xanthoparmelia* being the largest with over 800 accepted species [33]. The species in this genus characteristically occur on siliceous rocks or soil, predominantly in arid to subarid

regions, with a center of distribution in the southern hemisphere. The genus is characterized by having cell wall polysaccharides of the *Xanthoparmelia*-type, small ascospores with an arachiform vacuolar body [34], and the presence of a pored epicortex [33,35]. It has been hypothesized that the genus diversified in a rapid radiation following a shift towards drier habitats at the base of the *Xanthoparmelia* clade [36] leading to the high current diversity.

Previously, the *Xanthoparmelia pulla* group has been classified within the separate genus *Neofuscelia* based on the different cortical chemistry (having melanoid pigments and lacking usnic acid or atranorin, characteristic of the majority of *Xanthoparmelia* species) [37,38]. A subsequent molecular study showed that the genus *Neofuscelia* was polyphyletic, with its clades scattered within *Xanthoparmelia* [33]. Consequently, the genus *Neofuscelia* was reduced to synonymy with *Xanthoparmelia,* as have other genera previously distinguished by cortical chemistry or growth form [33,39–43]. The *Xanthoparmelia pulla* group is a monophyletic clade within the complete *Xanthoparmelia* clade, that includes the former Esslinger's *Xanthoparmelia pulla* species and other related species [44].

Although the clades were largely incongruent with the current species circumscription, we found a correlation of the main clades of *X. pulla* group with their geographical distribution and chemical profile (Fig. 1, 2). Clade 1 includes specimens from California, Macaronesia and areas around the Mediterranean basin, all of which contain depsides and depsidones derived from the orcinol pathway or with aliphatic acids; clade 2 includes specimens from Australia (subclade 2.1) and South America (subclade 2.2) with depsides and depsidones derived from the orcinol pathway; clade 3 derives from South African specimens containing olivetoric acid; clade 4 specimens with hypostictic acid from California (subclade 4.1) and South America (subclade 4.2); and clade 5 specimens with physodic acid (orcinol depsidones) from South Africa.

The species delimitation within the *Xanthoparmelia pulla* group is currently based on a combination of morphological and chemical characters (Table 1). The morphological characters include the color of the lower surface, shape of the lobes, attachment to the substrate, and presence of vegetative propagules while the chemical differences pertain to upper cortical and medullary secondary metabolites. A number of the currently accepted species have a wide distribution spanning several continents. To address the species delimitation in this group and to test the hypothesis of widely distributed species we have generated a data set using two loci (nuITS rDNA, nuLSU rDNA) from specimens collected on different continents. The molecular data were used to perform phylogenetic reconstructions in a maximum likelihood (ML) and Bayesian (B/MCMC) framework. We have also estimated the timing of the diversification events leading to the main clades found in our study to discriminate between vicariance and long-distance dispersal as possible explanations for the current distribution patterns. A Bayesian-based approach of ancestral range reconstruction was used to identify potential areas in which the group and major clades within the group originated.

## Results

### Phylogenetic analyses

One hundred sixty-eight DNA sequences of ITS and nuLSU rDNA of 88 representative specimens of *Xanthoparmelia* were assembled. One hundred forty of these sequences were newly generated in this study. The specimens included 25 currently accepted species in the *Xanthoparmelia pulla* group, four unassigned specimens, and six samples of four species as outgroup. A data matrix of 1283 unambiguously aligned characters, with 454 characters in the ITS and 829 characters in the nuLSU rDNA was used for phylogenetic analyses. The data set included 1081 constant characters. The general time-reversible model with a gamma distribution and invariant model of rate heterogeneity (GTR+I+G) was employed for analyses of the single-loci and concatenated data sets. Since no strongly supported conflicts between the two single-locus ML phylogenetic trees were detected, a combined data set was analyzed. In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following averaged values for the partitioned data set (± standard deviation): base frequencies $\pi$(A) = 0.25 (±1.54E-4), $\pi$(C) = 0.24 (±1.42E-4), $\pi$(G) = 0.28 (±1.58E-4), $\pi$(T) = 0.23 (±1.51E-4); rate matrix r(AC) = 4.42 (±1.43E-4), r(AG) = 0.23 (±8.35E-4), r(AT) = 9.53 (±2.19E-4), r(CG) = 4.82 (±1.45E-4), r(CT) = 0.54 (±9.26E-4), r(GT) = 2.98 (±1.11E-4) and the gamma shape parameter $\alpha$ = 0.21 (±3.86E-4). The likelihood parameters in the sample had a mean likelihood of LnL = −4608.25 (±0.49), while the ML tree had a likelihood of LnL = −4163.64.

The phylogenetic estimates of the ML and B/MCMC analyses were congruent, hence only the ML tree (Fig. 1) is shown here. Specimens of the *Xanthoparmelia pulla* group form a strongly supported monophyletic group with five main, mostly well-supported, clades (Fig. 1). The clades do not agree with the current species circumscription, with 11 species being polyphyletic, five of them with specimens from different continents entering different clades. For example, all the Northern Hemisphere specimens identified as *X. luteonotata*, *X. pulla*, *X. delisei* or *X. glabrans* belong to clade 1 while all the Australian specimens of the same species belong to clade 2.1. Similarly, specimens of *X. imitatrix* from South America belong to clade 2.2 and are not directly related to the South African specimen.

### Estimates of divergence times and ancestral range reconstructions

A Bayesian phylogenetic tree was dated to estimate the age of the *X. pulla* group and its main clades. The results of the divergence time analysis are summarized in Fig. 2, and the whole parmelioid tree is shown as supp. mat. (Fig. S1). The *Xanthoparmelia pulla* group started to diversify around 11.61 Ma (7.61 – 16.50 Ma), the age of the crown node of clade 1 was estimated at 5.31 Ma (3.01 – 8.38 Ma), the ancestor of clade 2 around 8.10 Ma (5.13 – 11.74 Ma), and the crown of clade 4 around 3.44 Ma (1.56 – 6.07 Ma).

The results of the ancestral range reconstruction analyses are summarized in Figs. 1 and 2. This established that South Africa was the most likely origin of the *X. pulla* group, with a marginal probability of 0.745, indicating localized uncertainty. The four other areas explored (South America, Australia, California and the Mediterranean basin) were rejected with probabilities below 0.05. For the base of clade 1, the Mediterranean basin was reconstructed as the most likely ancestral range with a marginal probability of 0.555, but California could not be rejected (probability of 0.104). For clade 2, Australia was recovered as the most likely ancestral area with marginal probability of 0.672; while South America, the other area from which specimens of this clade occur, was rejected as potential ancestral area ($p<0.05$); similarly, California, the Mediterranean basin and South Africa were also rejected as ancestral areas. South America was found to be the most likely origin for clade 4 (which also includes specimens from California and South America) with a marginal probability of 0.48, although neither California nor South Africa were rejected (probabilities of 0.106 and 0.083, respectively). Australia and the Mediterranean basin were rejected as ancestral ranges for clade 4.

**Table 1.** Main differences of the species of *Xanthoparmelia pulla* group studied in this paper [38,44,47,59,60].

| | Characters and distribution | | | | |
|---|---|---|---|---|---|
| **Species** | **Morphology of lobes** | **Isidia** | **Lower surface** | **Chemistry** | **Distribution** |
| *X. atroviridis* | 1–2 mm broad, subirregular, contiguous to imbricate | Absent | Black, moderately rhizinate, rhizines concolorous, up to 0.4 mm long. | Medulla: Hypoconstictic, hypostictic, hyposalazinic acids; Cortex: $HNO_3$ + violet | South Africa |
| *X. caliginosa* | 1–2.5 (−3.5) mm broad, subirregular, contiguous to imbricate | Sparse to crowded and areolate. Isidia erumpent, globose to cylindrical, 0.1–0.6 (−0.8) mm tall. | Dark brown to black, moderately rhizinate, rhizines more or less concolorous, up to 0.6 mm long. | Medulla: Olivetoric acid. Cortex: $HNO_3$ + blue-green | South Africa |
| *X. delisei* | 1–4 mm broad, sublinear to irregular, flat to slightly concave or convex, becoming laciniate, often imbricate and entangled | Absent | Dark brown to black, often paler near the apices, moderately to densely rhizinate, the rhizines simple and concolorous with the lower surface, to 1 mm long | Medulla: glomelliferic, glomellic, perlatolic acids; ± gyrophoric acid. Cortex: $HNO_3$ + blue-green | Europe, Asia, Africa, Australia, Macaronesia, South America |
| *X. fissurina* | 1–3 mm broad, contiguous to imbricate or entangled | Absent | Pale tan to pale brown, moderately rhizinate, rhizines concolorous, to 1 mm long | Medulla: hypostictic, hypoconstictic, hyposalazinic acids, unknown compounds. Cortex: $HNO_3$ + blue-green | South Africa and South America |
| *X. glabrans* | 0.5–3.0 mm broad, sublinear to linear-elongate, imbricate to loosely entangled. | Absent | black, dull, slightly rugulose, moderately rhizinate; rhizines black, simple or fasciculate, to 1 mm long | Medulla: alectoronic acid; ± a-collatolic and gyrophoric acids. Cortex: $HNO_3$ + blue-green | Australia, Europe, Africa, South America, New Zealand |
| *X. imitatrix* | 0.5–3.0 mm broad, sublinear to linear-elongate, imbricate to laciniate entangled, rarely developing subfruticose branches | Absent | Dark brown to black, sparsely to moderately rhizinate, rhizines simple, to 1.5 mm long | Medulla: physodic acid; ±4-O-methylphysodic and alectoronic acids. Cortex: $HNO_3$ + blue-green | Australia, Africa, South America, New Zealand |
| *X. lineella* | 0.1–0.5mm broad, linear and dichotomously branched and entangled | Absent | Black, sparsely rhizinate, rhizines concolorous, to 1 mm long | Medulla: physodic acid; Cortex: $HNO_3$ + blue-green | South Africa |
| *X. loxodes* | (0.5-)1–3(−5) mm broad, subirregular to sublinear, contiguous to entangled. | Sparsely to densely isidiate, isidia more or less spherical and distinctly pustular, erumpent | Dark brown to black, smooth to somewhat rugulose, moderately rhizinate, rhizines concolorous, to 1 mm long | Medulla: glomelliferic, glomellic and perlatolic acids; ±gyrophoric acid. Cortex: $HNO_3$ + blue-green | Europe, North Africa, Asia, North America, Macaronesia |
| *X. luteonotata* | (0.5-)1–3 mm broad, sublinear to irregular, discrete to imbricate, rarely developing subfruticose branches | Absent | Pale tan to pale brown, moderately to densely rhizinate, rhizines simple, to 0.5 mm long | Medulla: ± divaricatic and stenosporic acids; ± gyrophoric acid. Cortex: $HNO_3$ + blue-green | Australia, Europe, Africa, New Zealand |
| *X. pokornyi* | 1–2 mm broad, sublinear to linear, discrete to loosely imbricate or entangled | Absent | Pale tan to brown, moderately to sparsely rhizinate, rhizines concolorous or darkening, to 1 (−1.5) mm long | Medulla: stenosporic acid; ± gyrophoric and divaricatic acids. Cortex: $HNO_3$ + blue-green | Europe, Asia |
| *X. perrugata* | 1–3 (−5) mm broad, sublinear to linear-elongate, discrete to imbricate or entangled. | Absent | Dark brown to black, moderately to densely rhizinate, rhizines simple, to 1.5 mm long. | Medulla: divaricatic acid; ± stenosporic, oxostenosporic, gyrophoric, lecanoric acids. Cortex: $HNO_3$ + blue-green | Europe, North Africa, Australia, Asia |
| *X. pseudoglabrans* | 1–2.5 mm broad, subirregular to sublinear, imbricate to entangled | Absent | Black; moderately rhizinate or rhizines rather parse, concolorous with the lower surface | Medulla: alectoronic acid; ± a-collatolic acid. Cortex: $HNO_3$ - | South Africa |
| *X. pulla* | 1–3 (−5) mm broad, sublinear to linear-elongate, discrete to imbricate or entangled | Absent | Dark brown to black, moderately to densely rhizinate, rhizines simple, to 1.5 mm long | Medulla: stenosporic acid; ± divaricatic, gyrophoric, perlatolic, 4-O-demethylstenosporic acid, oxostenosporic acids. Cortex: $HNO_3$ + blue-green | Europe, Australia, New Zealand, Africa |

**Table 1.** Cont.

| | Characters and distribution | | | | |
|---|---|---|---|---|---|
| **Species** | **Morphology of lobes** | **Isidia** | **Lower surface** | **Chemistry** | **Distribution** |
| *X. pulloides* | 1–2 mm broad, subirregular to sublinear, contiguous to subimbricate | Absent | Black, moderately rhizinate, rhizines concolorous, to 0.5 mm long | Medulla: constipatic and protoconstipatic acids; ± gyrophoric acid. Cortex: $HNO_3$ + blue-green | Macaronesia, Asia |
| *X. quintarioides* | 1–2.5 (−3) mm broad, strongly convex and short-flabellate, discrete but close to more or less contiguous | Absent | Tan to pale brown, sparsely to moderately rhizinate, the rhizines short and hapterate | Medulla: hypostictic, hypoconstictic, cryptostictic acids; ± hyposalazinic acid. Cortex: $HNO_3$ + blue-green | South Africa |
| *X. ryssolea* | 1–3 mm broad, linear elongate, subterete, convex. | Absent | Pale yellow-brown to red-brown, canaliculate, sparsely rhizinate, rhizines concolorous, to 0.6 mm long. | Medulla: stenosporic acid; ± gyrophoric, oxostenosporic, divaricatic acids. $HNO_3$ + blue-green | Europe, Asia |
| *X. squamans* | 1–2 mm broad, sublinear, imbricate to contiguous | Absent | Dark brown to black, moderately to sparsely rhizinate, rhizines concolorous, to 1 mm long | Medulla: hypostictic, hypoconstictic, hyposalazinic acids. $HNO_3$ + blue-green | South Africa, South America, New Zealand |
| *X. subhosseana* | 1–2 mm broad, subirregular, contiguous to slightly imbricate | Sparsely to densely isidiate. Isidia pustular, erumpent | Dark brown to black, moderately rhizinate, rhizines concolorous, to 0.6 mm long | Medulla: hypostictic, hyposalazinic, hypoconstictic acids. Cortex: $HNO_3$ + blue-green | South Africa, North America, South America, New Zealand |
| *X. subimitatrix* | 0.5–2.0 mm broad, sublinear to subirregular, discrete to subimbricate | Absent | Pale tan to brown, moderately rhizinate, rhizines simple, brown or often blackened, to 0.8 mm long | Medulla: physodic acid and alectoronic acids. Cortex: $HNO_3$ + blue-green | South Africa, Australia. |
| *X. subincerta* | 0.5–1 mm broad, flat, sublinear, more or less imbricate | Isidia cylindrical, simple or densely branched, 0.08–0.5 mm tall. Apices syncorticate | Black, moderately rhizinate, rhizines simple, black, to 0.3 mm long | Medulla: glomelliferonic acid; ± loxodellonic and glomellonic acids. Cortex: $HNO_3$ + blue-green | Australia, South Africa |
| *X. subprolixa* | 1–3 (−5) mm broad, sublinear to linear-elongate, discrete to imbricate or entangled. | Absent | Dark brown to black, often paler at apices, moderately to densely rhizinate, rhizines simple, to 1.5 mm long | Medulla: divaricatic acid; ± stenosporic, nordivaricatic acids. Cortex: $HNO_3$ + blue-green | Australia, New Zealand |
| *X. torulosa* | 1.0–3.5 mm broad, sublinear to sublirregular, imbricate; laciniae at periphery and within thallus, ± subfruticose, sublinear to elongate, 0.3–1.0 mm broad. | Absent | Black, moderately to densely rhizinate; rhizines simple or occasionally tufted, slender. | Medulla: divaricatic acid; ± nordivaricatic, stenosporic acids. Cortex: $HNO_3$ + blue-green | Australia |
| *X. verisidiosa* | 1–3 mm broad, irregular to sublinear, flat, short and rounded, contiguous to imbricate | Sparsely to densely isidiate. Isidia cylindrical, simple or becoming densely branched, 0.2–1 mm tall. Apices syncorticate | Black, moderately to sparsely rhizinate, rhizines simple, black to 0.4 mm long | Medulla: alectoronic and a-collatolic acids. Cortex: $HNO_3$ + blue-green | Australia, New Zealand, South Africa |
| *X. verrucella* | 0.5–2 mm wide, irregular to sublinear, flat, imbricate to entangled. | Moderate to densely isidiate. Isidia cylindrical, simple or becoming branched, to 1 mm tall. Apices syncorticate | Black, sparsely to moderately rhizinate, rhizines simple, simple, black, to 0.4 mm long. | Medulla: divaricatic acid; ± stenosporic acid. Cortex: $HNO_3$ + blue-green | Australia, New Zealand, South Africa |
| *X. verruculifera* | 1–2 mm broad, subirregular to sublinear, contiguous to imbricate | Sparcely to densely isidiate. Isidia pustular, erumpent. | Dark brown to black, moderately rhizinate, rhizines concolorous, to 0.8 mm long | Medulla: divaricatic acid; ± stenosporic and gyrophoric acids. Cortex: $HNO_3$ + blue-green | North Africa, Europe, North America |

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## Discussion

Understanding the diversity and delimiting species in lichenized fungi has been a long standing challenge and current studies using molecular data have dramatically changed our ability to distinguish species in this group [8,23,45]. The *Xanthoparmelia pulla* group is a good example for illustrating the difficulties in in distinguishing species by morphology due to the remarkable

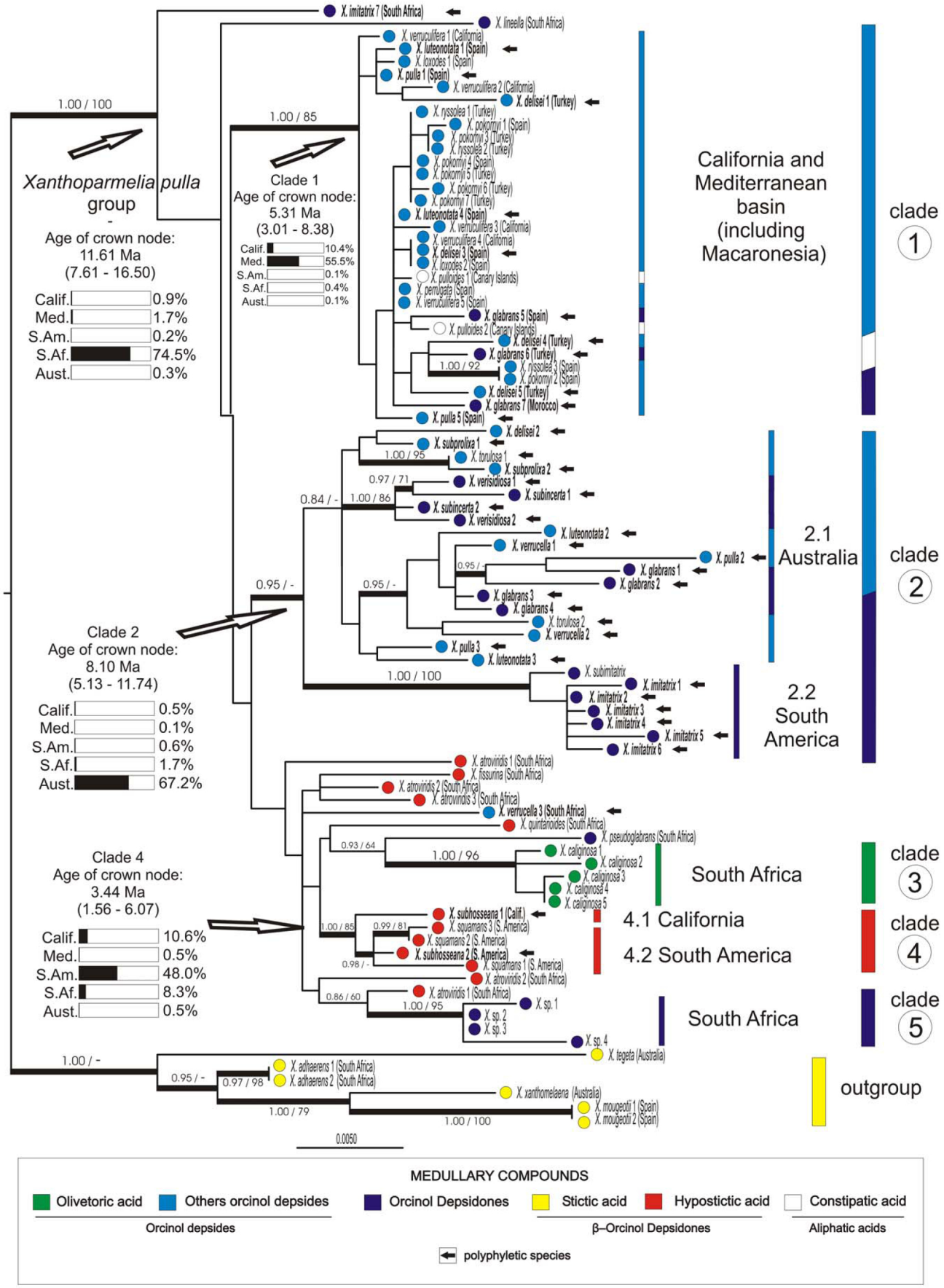
X. imitatrix 7 (South Africa)
X. lineella (South Africa)
X. verruculifera 1 (California)
X. luteonotata 1 (Spain)
X. loxodes 1 (Spain)
X. pulla 1 (Spain)
X. verruculifera 2 (California)
X. delisei 1 (Turkey)
X. ryssolea 1 (Turkey)
X. pokornyi 1 (Spain)
X. pokornyi 3 (Turkey)
X. ryssolea 2 (Turkey)
X. pokornyi 4 (Spain)
X. pokornyi 5 (Turkey)
X. pokornyi 6 (Turkey)
X. pokornyi 7 (Turkey)
X. luteonotata 4 (Spain)
X. verruculifera 3 (California)
X. verruculifera 4 (California)
X. delisei 3 (Spain)
X. loxodes 2 (Spain)
X. pulloides 1 (Canary Islands)
X. perrugata (Spain)
X. verruculifera 5 (Spain)
X. glabrans 5 (Spain)
X. pulloides 2 (Canary Islands)
X. delisei 4 (Turkey)
X. glabrans 6 (Turkey)
X. ryssolea 3 (Spain)
X. pokornyi 2 (Spain)
X. delisei 5 (Turkey)
X. glabrans 7 (Morocco)
X. pulla 5 (Spain)
X. delisei 2
X. subprolixa 1
X. torulosa 1
X. subprolixa 2
X. verisidiosa 1
X. subincerta 1
X. subincerta 2
X. verisidiosa 2
X. luteonotata 2
X. verrucella 1
X. pulla 2
X. glabrans 1
X. glabrans 2
X. glabrans 3
X. glabrans 4
X. torulosa 2
X. verrucella 2
X. pulla 3
X. luteonotata 3
X. subimitatrix
X. imitatrix 1
X. imitatrix 2
X. imitatrix 3
X. imitatrix 4
X. imitatrix 5
X. imitatrix 6
X. atrovirids 1 (South Africa)
X. fissurina (South Africa)
X. atrovirids 2 (South Africa)
X. atrovirids 3 (South Africa)
X. verrucella 3 (South Africa)
X. quintarioides (South Africa)
X. pseudoglabrans (South Africa)
X. caliginosa 1
X. caliginosa 2
X. caliginosa 3
X. caliginosa 4
X. caliginosa 5
X. subhosseana 1 (Calif.)
X. squamans 3 (S. America)
X. squamans 2 (S. America)
X. subhosseana 2 (S. America)
X. squamans 1 (S. America)
X. atrovirids 2 (South Africa)
X. atrovirids 1 (South Africa)
X. sp. 1
X. sp. 2
X. sp. 3
X. sp. 4
X. tegeta (Australia)
X. adhaerens 1 (South Africa)
X. adhaerens 2 (South Africa)
X. xanthomelaena (Australia)
X. mougeotii 1 (Spain)
X. mougeotii 2 (Spain)
1.00 / 100
1.00 / 85
1.00 / 92
1.00 / 95
0.97 / 71
0.84 / -
1.00 / 86
0.95 / -
0.95 / -
0.95 / -
1.00 / 100
0.93 / 64
1.00 / 96
1.00 / 85
0.99 / 81
0.98 / -
0.86 / 60
1.00 / 95
1.00 / -
0.95 / -
0.97 / 98
1.00 / 79
1.00 / 100
0.0050
Xanthoparmelia pulla group
Age of crown node: 11.61 Ma (7.61 - 16.50)
Calif. 0.9%
Med. 1.7%
S.Am. 0.2%
S.Af. 74.5%
Aust. 0.3%
Clade 1
Age of crown node: 5.31 Ma (3.01 - 8.38)
Calif. 10.4%
Med. 55.5%
S.Am. 0.1%
S.Af. 0.4%
Aust. 0.1%
Clade 2
Age of crown node: 8.10 Ma (5.13 - 11.74)
Calif. 0.5%
Med. 0.1%
S.Am. 0.6%
S.Af. 1.7%
Aust. 67.2%
Clade 4
Age of crown node: 3.44 Ma (1.56 - 6.07)
Calif. 10.6%
Med. 0.5%
S.Am. 48.0%
S.Af. 8.3%
Aust. 0.5%
California and Mediterranean basin (including Macaronesia)
clade 1
2.1 Australia
clade 2
2.2 South America
South Africa
clade 3
4.1 California
4.2 South America
clade 4
South Africa
clade 5
outgroup
MEDULLARY COMPOUNDS
Olivetoric acid
Others orcinol depsides
Orcinol depsides
Orcinol Depsidones
Stictic acid
Hypostictic acid
β–Orcinol Depsidones
Constipatic acid
Aliphatic acids
polyphyletic species

**Figure 1. Phylogenetic relationships of the *Xanthoparmelia pulla* group based on nuITS and nuLSU rDNA sequences.** Topology based on maximum-likelihood (ML) analyses. Posterior probabilities and bootstrap values are indicated on each branch. Branches with posterior probabilities under Bayesian analysis equal or above 0.95 and/or bootstrap values equal or above 70% under MP are in bold. Medullary compounds, and results of the divergence time estimation and ancestral range reconstruction analyses are shown. Black arrow head indicates the polyphyletic species.
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plasticity of morphological characters in this group. Consequently, secondary metabolites have played an important role in delimiting species in this group [44,46,47]. Following the current classification using a combination of vegetative morphology and secondary chemistry, a number of species have broad geographical distributions spanning several continents.

Here we have used molecular data to investigate the current classification within the group and attempt to explain their distribution. We used likelihood-based and Bayesian approaches to investigate the evolutionary origin of the group and timing of speciation events. Hopefully such data will reveal evolutionary patterns so we may develop a framework for their taxonomic classification which better reflects the phylogenetic relationships in the *X. pulla* group. Our results clearly indicate that the species as currently delimited are polyphyletic (Fig. 1). This is consistent with results from other studies of *Xanthoparmelia* species believed to occur on different continents which were subsequently found to represent distinct lineages [15,16]. Further, similar patterns have been found in other groups of lichenized fungi [4,14,18,48].

The ancestral range reconstruction points to South Africa as the most likely origin of the *X. pulla* group. Although there is a certain degree of uncertainty in the reconstruction (marginal probability of 0.745), the analysis rejected other areas as potential ancestral areas for the group. Interestingly, South Africa has the highest morphological and chemical diversity within the group and the specimens studied here belong to different, unrelated lineages (Fig. 1). South African specimens containing olivetoric acid cluster in clade 3 and those with physodic acid in clade 5. The phylogenetic relationships of other specimens from South Africa with hypostictic acid, physodic acid or other orcinol depsides and depsidones are still unresolved. The South African specimens show remarkable morphological variability, including subcrustose and foliose species. Further, many *Xanthoparmelia* species occur in arid climates and the diversification of *X. pulla* group occurred around 11.61 Ma (7.61 – 16.50). At this time the Cape Region underwent a major aridification [49], which may be responsible for the rapid radiation and current richness of the Cape flora. Thus, it is likely that the *X. pulla* group originated in South Africa around the same time. Unlike most Cape region elements in flowering plants, the species of the *X. pulla* group subsequently extended their distribution by transoceanic dispersal.

Within the *X. pulla* group, the five lineages identified are characterized by the presence of different substance classes and in some cases they diverged secondarily in different geographical areas. The correlation between chemical pathways and the lineages found in molecular studies has also been found in Pertusariaceae among lichenized fungi [50–52].

Clade 1 includes specimens containing orcinol depsides and depsidones that occur in California and the Mediterranean basin. Neither internally supported subclades nor a geographical pattern was found within this clade, and specimens with different phenotypical characters from different geographical areas are

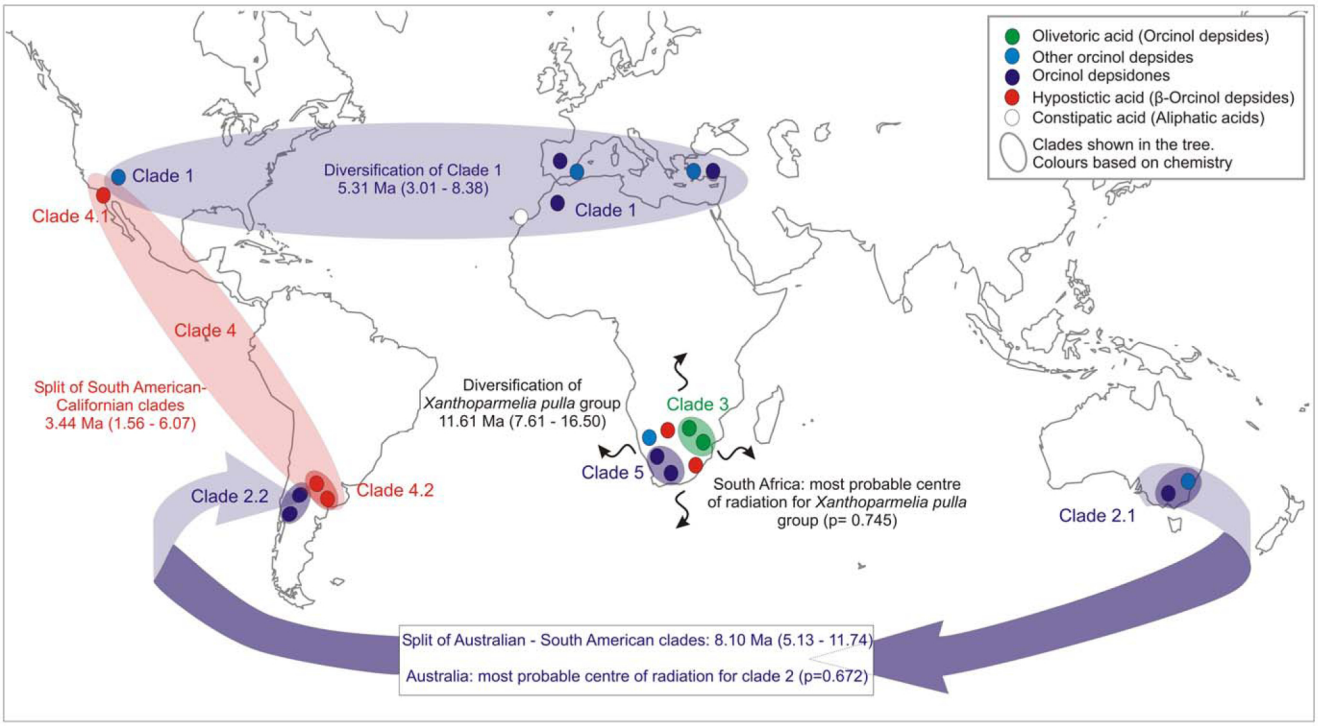


**Figure 2. Schematic map showing the relationships between phylogeny, medullary compounds, ancestral range and divergence times estimation for the *Xanthoparmelia pulla* group.**
doi:10.1371/journal.pone.0039683.g002

**Table 2.** Specimens used in this study with country of collection, voucher information and GenBank accession numbers.

| | | | GenBank accession no. | |
|---|---|---|---|---|
| **Species** | **Country** | **Herbarium acc. no.** | **nuITS** | **nuLSU** |
| *Xanthoparmelia adhaerens* 1 | South Africa | MAF-Lich 16212 | HM125744 | HM125766 |
| *X. adhaerens* 2 | South Africa | MAF-Lich 16213 | HM125746 | HM125768 |
| *X. atroviridis* 1 | South Africa | MAF-Lich 17163 | **JQ912329** | **JQ912425** |
| *X. atroviridis* 1 | South Africa | MAF-Lich 17168 | **JQ912351** | **JQ912448** |
| *X. atroviridis* 2 | South Africa | MAF-Lich 17158 | **JQ912314** | **JQ912415** |
| *X. atroviridis* 2 | South Africa | MAF-Lich 17154 | **JQ912320** | **JQ912419** |
| *X. atroviridis* 3 | South Africa | MAF-Lich 17153 | **JQ912349** | **JQ912446** |
| *X. caliginosa* 1 | South Africa | MAF-Lich 17157 | **JQ912350** | **JQ912447** |
| *X. caliginosa* 2 | South Africa | MAF-Lich 17150 | **JQ912315** | - |
| *X. caliginosa* 3 | South Africa | MAF-Lich 17156 | **JQ912333** | **JQ912430** |
| *X. caliginosa* 4 | South Africa | MAF-Lich 17152 | **JQ912317** | - |
| *X. caliginosa* 5 | South Africa | MAF-Lich 17186 | **JQ912348** | **JQ912445** |
| *X. delisei* 1 | Turkey | MAF-Lich 17139 | **JQ912307** | **JQ912408** |
| *X. delisei* 2 | Australia | MAF-Lich 7432 | AY581067 | AY578930 |
| *X. delisei* 3 | Spain | MAF-Lich 7659 | AY581068 | AY578931 |
| *X. delisei* 4 | Turkey | MAF-Lich 17134 | **JQ912308** | **JQ912409** |
| *X. delisei* 5 | Turkey | MAF-Lich 17135 | **JQ912305** | **JQ912406** |
| *X. fissurina* | South Africa | MAF-Lich 17162 | **JQ912353** | **JQ912450** |
| *X. glabrans* 1 | Australia | CANB 746334 | **JQ912291** | **JQ912393** |
| *X. glabrans* 2 | Australia | CANB 746340 | **JQ912289** | **JQ912391** |
| *X. glabrans* 3 | Australia | MAF-Lich 7665 | AY581069 | AY578932 |
| *X. glabrans* 4 | Australia | CANB 681875.1 | **JQ912290** | **JQ912392** |
| *X. glabrans* 5 | Spain | MAF-Lich 9912 | AY581072 | AY578935 |
| *X. glabrans* 6 | Turkey | MAF-Lich 17137 | **JQ912306** | **JQ912407** |
| *X. glabrans* 7 | Morocco | MAF-Lich 17144 | **JQ912286** | **JQ912388** |
| *X. imitatrix* 1 | Chile | MAF-Lich 17132 | **JQ912344** | **JQ912441** |
| *X. imitatrix* 2 | Chile | MAF-Lich 17126 | **JQ912288** | **JQ912390** |
| *X. imitatrix* 3 | Chile | MAF-Lich 17123 | **JQ912287** | **JQ912389** |
| *X. imitatrix* 4 | Chile | MAF-Lich 17127 | **JQ912342** | **JQ912439** |
| *X. imitatrix* 5 | Chile | MAF-Lich 17122 | **JQ912285** | **JQ912387** |
| *X. imitatrix* 6 | Chile | MAF-Lich 17124 | **JQ912326** | **JQ912422** |
| *X. imitatrix* 7 | South Africa | MAF-Lich 17155 | **JQ912352** | **JQ912449** |
| *X. lineella* | South Africa | MAF-Lich 17160 | **JQ912319** | **JQ912418** |
| *X. loxodes* 1 | Spain | MAF-Lich 7072 | AY581076 | AY578940 |
| *X. loxodes* 2 | Spain | MAF-Lich 6206 | AY581070 | AY578933 |
| *X. luteonotata* 1 | Spain | MAF-Lich 17120 | **JQ912341** | **JQ912438** |
| *X. luteonotata* 2 | Australia | CANB 746358 | **JQ912293** | - |
| *X. luteonotata* 3 | Australia | CANB 746366.1 | **JQ912292** | **JQ912394** |
| *X. luteonotata* 4 | Spain | MAF-Lich 17119 | **JQ912340** | **JQ912437** |
| *X. mougeotii* 1 | Spain | MAF-Lich 6802 | AY37006 | AY578966 |
| *X. mougeotii* 2 | Spain | MAF-Lich 9916 | AY581100 | AY578967 |
| *X. perrugata* | Spain | MAF-Lich 17118 | **JQ912324** | - |
| *X. pokornyi* 1 | Spain | MAF-Lich 6052 | AY037005 | AY578934 |
| *X. pokornyi* 2 | Spain | MAF-Lich 9908 | AY581075 | AY578939 |
| *X. pokornyi* 3 | Turkey | MAF-Lich 17140 | **JQ912310** | **JQ912411** |
| *X. pokornyi* 4 | Spain | MAF-Lich 17117 | **JQ912323** | - |
| *X. pokornyi* 5 | Turkey | MAF-Lich 17143 | **JQ912313** | **JQ912414** |
| *X. pokornyi* 6 | Turkey | MAF-Lich 17142 | **JQ912312** | **JQ912413** |
| *X. pokornyi* 7 | Turkey | MAF-Lich 17136 | **JQ912304** | **JQ912405** |

**Table 2.** Cont.

| | | | GenBank accession no. | |
|---|---|---|---|---|
| **Species** | **Country** | **Herbarium acc. no.** | **nuITS** | **nuLSU** |
| *X. pseudoglabrans* | South Africa | MAF-Lich 17161 | **JQ912316** | **JQ912416** |
| *X. pulla* 1 | Spain | MAF-Lich 17115 | - | **JQ912420** |
| *X. pulla* 2 | Australia | CANB 739130.1 | **JQ912294** | **JQ912395** |
| *X. pulla* 3 | Australia | CBG 9810185 | **JQ912295** | **JQ912396** |
| *X. pulla* 5 | Spain | MAF-Lich 6794 | AY581071 | AJ 421433 |
| *X. pulloides* 1 | Spain | MAF-Lich 17121 | **JQ912347** | **JQ912444** |
| *X. pulloides* 2 | Spain | MAF-Lich 6784 | AY037004 | AY578936 |
| *X. quintarioides* | South Africa | MAF-Lich 17159 | **JQ912318** | **JQ912417** |
| *X. ryssolea* 1 | Turkey | MAF-Lich 17141 | **JQ912311** | **JQ912412** |
| *X. ryssolea* 2 | Turkey | MAF-Lich 17138 | **JQ912309** | **JQ912410** |
| *X. ryssolea* 3 | Spain | MAF-Lich 17116 | **JQ912322** | - |
| *X. sp.* 1 | South Africa | MAF-Lich 17166 | **JQ912330** | **JQ912426** |
| *X. sp.* 2 | South Africa | MAF-Lich 17167 | **JQ912331** | **JQ912427** |
| *X. sp.* 3 | South Africa | MAF-Lich 17165 | - | **JQ912429** |
| *X. sp.* 4 | South Africa | MAF-Lich 17164 | **JQ912339** | **JQ912436** |
| *X. squamans* 1 | Chile | MAF-Lich 17128 | **JQ912325** | **JQ912421** |
| *X. squamans* 2 | Chile | MAF-Lich 17129 | **JQ912327** | **JQ912423** |
| *X. squamans* 3 | Chile | MAF-Lich 17131 | **JQ912343** | **JQ912440** |
| *X. subhosseana* 1 | USA | MAF-Lich 17149 | **JQ912337** | **JQ912434** |
| *X. subhosseana* 2 | Chile | MAF-Lich 17133 | **JQ912345** | **JQ912442** |
| *X. subimitatrix* | Chile | MAF-Lich 17130 | **JQ912328** | **JQ912424** |
| *X. subincerta* 1 | Australia | CANB 746346.1 | **JQ912296** | **JQ912397** |
| *X. subincerta* 2 | Australia | MAF-Lich 7494 | AY581073 | AY578937 |
| *X. subprolixa* 1 | Australia | MAF-Lich 7667 | AY581074 | AY578938 |
| *X. subprolixa* 2 | Australia | CANB 746355 | **JQ912297** | **JQ912398** |
| *X. tegeta* | Australia | MAF-Lich 7523 | AY581107 | AY578975 |
| *X. torulosa* 1 | Australia | CANB 746363.1 | **JQ912299** | **JQ912400** |
| *X. torulosa* 2 | Australia | CANB 746351 | **JQ912298** | **JQ912399** |
| *X. verisidiosa* 1 | Australia | CANB 746341.1 | **JQ912301** | **JQ912402** |
| *X. verisidiosa* 2 | Australia | CANB 746345.1 | **JQ912300** | **JQ912401** |
| *X. verrucella* 1 | Australia | CANB 746353 | **JQ912303** | **JQ912404** |
| *X. verrucella* 2 | Australia | CANB 746349 | **JQ912302** | **JQ912403** |
| *X. verrucella* 3 | South Africa | MAF-Lich 17151 | **JQ912332** | **JQ912428** |
| *X. verruculifera* 1 | USA | MAF-Lich 17146 | **JQ912334** | **JQ912431** |
| *X. verruculifera* 2 | USA | MAF-Lich 17147 | **JQ912336** | **JQ912433** |
| *X. verruculifera* 3 | USA | MAF-Lich 17145 | **JQ912338** | **JQ912435** |
| *X. verruculifera* 4 | USA | MAF-Lich 17148 | **JQ912335** | **JQ912432** |
| *X. verruculifera* 5 | Spain | MAF-Lich 17114 | **JQ912321** | - |
| *X. xanthomelaena* | Australia | MAF-Lich 16447 | HM125740 | HM125761 |

New sequences are in bold.

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intermingled. In fact, shifting between orcinol depsides and depsidones can occur by one-step transformations [46]. By contrast, in other genera (e.g. *Melanelixia*, *Parmelina*, *Leptogium*) with similar disjunct distributions (North America and the Mediterranean basin), the geographical distribution correlates with the clades found in the molecular study. In the *X. pulla* group this pattern was not found, possibly due to insufficient sampling or absence of a phylogenetic signal in the markers used. This might be due to the slower evolutionary rates of lichenized fungi from arid and subarid regions compared to oceanic parmelioid lichens [36]. The diversification age of clade 1 was estimated at 5.31 Ma (3.01 – 8.38 Ma), at the end of the Miocene, which was a geological period when numerous groups radiated in arid conditions. The Mediterranean region was suggested as the ancestral area of this clade by the ancestral range analysis, but the result was poorly supported.

Clade 2 also contains species with orcinol depsides and depsidones and comprises two disjunct lineages, one occurring in Australia (clade 2.1) and the other in South America (2.2). The estimated age for clade 2 (8.10 Ma; 5.13 – 11.74 Ma) rules out the possibility of vicariance, since the breakup of Australia, Antarctica and South America occurred between 35–52 Ma ago [53]. The ancestral range reconstruction points to Australia as the ancestral area of clade 2. This would be consistent with long distance dispersal from Australia to South America, a phenomenon frequently found in many plants groups [54]. Within the Australian clade several strongly supported lineages are not consistent with the current species delimitation of the group, indicating that the phenotypical characters used to distinguish species in the group have limited phylogenetic validity. Similar disparities between phylogenetic relationships and current species delimitations were found within the yellow *Xanthoparmelia* species from western North American [55,56].

Clade 4 includes specimens containing hypostictic acid from California and South America. Here again all the South American specimens form a monophyletic group. Specimens of *X. subhosseana* occurring in different continents are not closely related. The most likely ancestral origin of clade 4 is South America (marginal probability of 0.48), although neither California nor South Africa could be rejected.

Our study indicates that the *X. pulla* group started to radiate during the Miocene in South Africa, where the highest diversity of this group is found. From this region, different lineages with distinct secondary metabolites belonging to different chemical pathways were dispersed to other regions, where they experienced rapid and more recent radiations. In some cases our results showed that the sympatric species of the *X. pulla* group in an area belong to distantly related groups. For example, the Californian *X. pulla* flora includes species from clade 1 and clade 4.1, the latter most probably having migrated from South America. Indeed our study indicated that the current taxonomic circumscription of species in the group does not agree with the evolutionary hypotheses inferred by molecular markers. The incongruence of phenotype-based classification and molecular phylogeny is a challenge for the classification of these fungi. Additional studies will be needed to determine whether the lineages found here represent cryptic species or whether new phenotypical characters can be found to distinguish these distinct lineages (as has been found in some other Parmeliaceae [10,57]). Future research should address how such parallel evolution of phenotypical characters in lichenized fungi could be explained in order to provide a better framework to test the adaptive value of these characters [58]. Our results here have important implications for conservation and ecological issues, since species were found to have much more restricted distribution than previously thought.

## Materials and Methods

### Taxon sampling

Eighty two specimens of the *Xanthoparmelia pulla* group from California, the Mediterranean basin, Macaronesia, South America, Australia and South Africa were used for the phylogenetic study. The specimens were identified following the current species delimitations [38,44,47,59,60]. Chemical constituents were identified using thin layer chromatography (TLC) [61–64], and gradient-elution high performance liquid chromatography (HPLC) [65]. The major medullary compounds were classified into four major groups based on their chemical structure: 1) Orcinol depsides: olivetoric, divaricatic, stenosporic and glomelliferic acids. 2) Orcinol depsidones, physodic, alectoronic and glomelliferonic acids. 3) β-Orcinol depsidones: stictic acid (only present in the outgroup) and hypostictic acid. 4) aliphatic acids: constipatic acid. All necessary permits were obtained for the described field studies. Collecting permits in Australia were all obtained by J.A. Elix (ca. 50 permit numbers for each states and over several years) and in Chile by W. Quilhot. For European locations specific permission was not required, since the locations were neither in privately-owned or protected areas. The field studies did not involve endangered or protected species.

### Molecular study

Total DNA was extracted from frozen lobes of thalli crushed with sterile glass pestles, using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions and modifications of Crespo et al. [66]. The following primers were used: ITS1-LM [67] and ITS2-KL [68] for nuITS rDNA, and LR0R and LR5 [69] for nuLSU rDNA.

For each amplification we used a reaction mixture of 25 μL, containing: 2.5 μL of 10x DNA polymerase buffer (including $MgCl_2$ 2mM) (Biotools), 1.25 μL of each primer, 0.75 μL of DNA polymerase (1U/μL), 0.5 μL of dNTPs containing 10 mM of each base (Biotools), 5 μL of DNA (third elution of DNA extraction) and 13.5 μL $dH_2O$. Amplifications were carried out in an automatic thermocycler (Techne Progene 3000) with the following steps: an initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, 58°C (nuITS rDNA) or 56°C (nuLSU rDNA) for 1 min, and 72°C for 1.5 min; a final extension at 72°C for 5 min. PCR products were cleaned with DNA Purification Kit (Flavorgen) and sequenced with the same primers using the ABI Prism Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) with the following program: initial denaturation at 94°C for 3 min, 25 cycles at 96°C for 10s, 50°C for 5s and 60°C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems). The sequence fragments were assembled with Bioedit v. 7.0 [70] and manually adjusted.

### Sequence alignment and selection of the substitution model

We used a dataset of 2 loci of 82 specimens representing 25 species of the *Xanthoparmelia pulla* group and 6 specimens as outgroup. The sequences were mainly generated in this study (140 sequences) and the others taken from our previous studies [33,41]. The outgroup selection was based on previous phylogenetic studies [41]. GenBank accession numbers and sources of the specimens are listed in Table 2.

The two loci were aligned separately with Muscle 3.6 [71] and the ambiguous positions were removed manually. The general time reversible model including estimation of invariant sites (GTR+I+G) was selected by jModelTest v 0.1.1 [72] as the most appropriate nucleotide substitution model for both loci.

### Phylogenetic Analyses

Potential conflict between the two loci was assessed by comparison of the ML analyses obtained with Garli 0.96 [73] for each locus, using 100 pseudoreplicates for the bootstrap analyses. The phylogenetic analyses of the combined matrix were done using maximum likelihood (ML) and a Bayesian approach. ML analysis was performed using Garli 0.96 [73] with default settings and 100 replicates for the bootstrap analyses. The Bayesian analysis was performed using MrBayes 3.1.1 [74] using the GTR+I+G model, and the data set partitioned into nu ITS and nu LSU. Each partition was allowed to have its own parameter values [75]. Heating of chains was set to 0.2, with 5

million generations sampled every 500th tree. The first 1000 trees were discarded as burn in. We used AWTY [76] to compare splits frequencies in the different runs and to plot cumulative split frequencies to insure that stationarity was reached. Of the remaining 18000 trees (9000 from each of the parallel runs) a majority rule consensus tree with average branch lengths and posterior probabilities was calculated using the sumt option of MrBayes. Clades with bootstrap support equal or above 70 % under ML and/or posterior probabilities ≥0.95 in the Bayesian analysis were considered as strongly supported. Phylogenetic trees were visualized using the program FigTree [77].

### Calibration of nodes and dating analysis

The ages of the *X. pulla* group and its major clades were estimated by a divergence time analysis based on a calibrated phylogeny of the parmelioid lichens [78]. We used a matrix of two loci (nu ITS and LSU) with a proportional number of samples of each parmelioid clade to have a representative tree and trend in speciation through time [79]. The matrix included 299 specimens of parmelioid lichens and 3 specimens of the genus *Usnea* (as outgroup); 62 new sequences of *Xanthoparmelia* species outside the *X. pulla* group were included. GenBank accession numbers with the specimens of the dating analysis are listed in Table S1. The sequence alignment, selection of the nucleotide substitution model, and phylogenetic analyses were done using the same procedures used for the *Xanthoparmelia pulla* dataset (see above).

The divergence time analyses were performed using BEAST v.1.6.1 [80]. We used a starting tree obtained from a ML analysis using Garli 0. 96 [73] of the concatenated dataset, calculated an ultrametric tree using nonparametric rate smoothing (NPRS) implemented in TreeEdit v.10a10 [81]. The age of the crown node of the parmelioid lichens was calibrated at 60 Ma, following Amo de Paz et al. [78]. The starting tree was topologically congruent with the parmelioid phylogeny presented in Crespo et al. [32].

For the divergence time analyses we used two points of calibration: the age of the crown node of the parmelioid lichens set at 60.28 Ma (49.81 – 73.55 Ma) [78], and the age of the crown node of the genus *Parmelia* (dated from the fossil *Parmelia ambra* from the Dominican amber, 15–45 Ma, [82] as discussed previously) [78].

The BEAST analysis was performed using the GTR+I+G substitution model, a Birth-Death process tree prior, and a relaxed clock model (uncorrelated lognormal) for the concatenated dataset. Calibration points were defined as prior distribution, minimal ages and calibrated with a lognormal distributions: 1) the parmelioid crown node at uniform distribution between 49 – 73 Ma; 2) the *Parmelia* crown node at log-normal mean = 2.77, offset = 14, lognormal standard deviation = 0.5. The analysis was run for 10 million generations, with parameter values sampled every 1000 generation. We checked the stationary plateau with Tracer v. 1.5 [83]. We discarded 10% of the initial trees as burn in and the consensus tree was calculated using Tree Annotator v 1.6.1 [80]. The results were visualized with FigTree v. 1.3.1 [84]. Ages of the *X. pulla* clades were estimated for nodes with more than 0.95 of posterior probability in the BEAST runs and in the previous Bayesian analysis.

### Ancestral range reconstructions

The biogeographical analysis to reconstruct the ancestral area was performed using an indirect Bayesian approach to character state reconstruction [85] implemented in SIMMAP v1.5 [86] following [87]. This analysis integrates the combination of the uncertainty in the tree, branch lengths and the substitution models using Markov chain Monte Carlo. We treated the biogeographic regions as discrete characters. The major areas in which *X. pulla* species are distributed were categorised broadly into five areas: California, Mediterranean basin (including Macaronesia), South America, South Africa, and Australia. Presence/absence was coded as binary states and each area was given equal probability. We performed the ancestral state reconstruction analysis on a sub-sample of 1000 trees derived from the MrBayes tree sampling of the *Xanthoparmelia pulla* group.

We also performed ancestral range reconstruction analysis using dispersal-extinction-cladogenesis (DEC) implemented in Lagrange program [88]. The results were inconclusive due to the lack of confidence in parts of the *X. pulla* phylogeny and hence the results are not included in this paper.

## Supporting Information

**Figure S1 Chronogram of parmelioid lichens focusing in *Xanthoparmelia pulla* group**. Calibration points: A, inferred age of radiation of parmelioid lichens and B, age of the *Parmelia* fossil. The *Xanthoparmelia pulla* group is highlighted by a box and the dated clades are indicated by a branch in bold.
(TIF)

**Table S1 GenBank accession numbers of parmelioid lichens (except X. pulla group, see Table 2) used for divergence time analysis**. New sequences are in bold.
(DOC)

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## Author Contributions

Conceived and designed the experiments: HTL AC PC. Performed the experiments: GAP. Analyzed the data: HTL PC GAP. Contributed reagents/materials/analysis tools: JAE GAP. Wrote the paper: HTL AC PC GAP.

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**Supporting information**

**Fig. S1**. **Chronogram of parmelioid lichens focusing in *Xanthoparmelia pulla* group**. Calibration points: A, inferred age of radiation of parmelioid lichens and B, age of the *Parmelia* fossil. The *Xanthoparmelia pulla* group is highlighted by a box and the dated clades are indicated by a branch in bold.

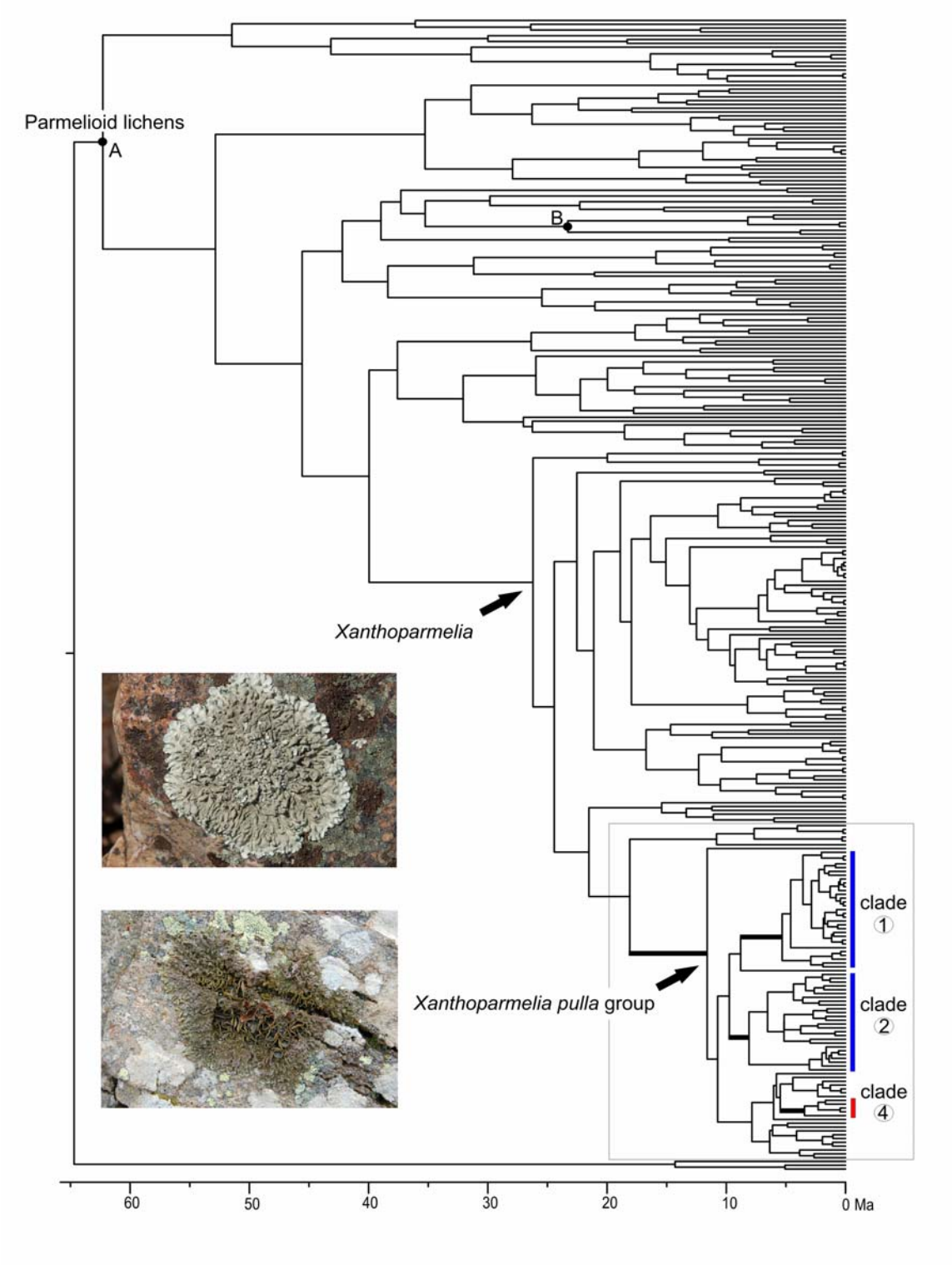

**Table S1. GenBank accession numbers of parmelioid lichens (except *X. pulla* group, see Table 2) used for divergence time analysis.** New sequences are in bold.

| | GenBank Acc. No. | |
|---|---|---|
| species | nuITS | nuLSU |
| *Austroparmelina pseudorelicina* | GU994540 | GU994583 |
| *Bulbothrix apophysata* | DQ279481 | EU562670 |
| *B. coronata* | DQ279482 | EU562671 |
| *B. decurtata* | DQ279483 | EU562672 |
| *B. goebelii* | DQ279484 | EU562673 |
| *B. meizospora* | AY611068 | AY607780 |
| *B. setschwanensis* | AY611069 | AY607781 |
| *Canoparmelia crozalsiana* | AY586571 | AY584831 |
| *Cetrariastrum dubitans* | GQ919270 | GQ919246 |
| *Cetrelia chicitae* | AF451759 | DQ923658 |
| *C. olivetorum* | AF451763 | DQ923659 |
| *Everniastrum cirrhatum* | DQ279487 | AY607782 |
| *E. lipidiferum* | DQ279488 | EU562675 |
| *E. nepalense* | DQ383642 | AY607783 |
| *E. rhizodendroideum* | DQ279489 | EU562676 |
| *E. sorocheilum* | DQ279490 | EU562677 |
| *E. vexans* | DQ279491 | EU562678 |
| *Flavoparmelia baltimorensis* | AY586559 | AY584833 |
| *F. caperata* | AY586561 | AY584639 |
| *F. citrinescens* | GU994550 | GU994596 |
| *F. haysomii* | DQ299904 | GU994597 |
| *F. marchantii* | DQ299905 | GU994598 |
| *F. soredians* | AY586562 | AY584835 |
| *F. springtonensis* | EF042907 | EF042916 |
| *F. subambigua* | GU994551 | GU994599 |
| *Flavopunctelia flaventior* | AY773127 | DQ912335 |
| *F. flaventior* | AY773126 | AY578923 |
| *F. soredica* | AY773128 | GU994600 |
| *Hypotrachyna aff taylorensis* | DQ279529 | EU562681 |
| *H. booralensis* | DQ279493 | EU562682 |
| *H. endochlora* | DQ279496 | AY607784 |
| *H. exsecta* | DQ279498 | EU562684 |
| *H. imbricatula* | DQ279503 | EU562686 |
| *H. immaculata* | DQ279504 | AY607785 |
| *H. laevigata* | AY611074 | AY607786 |
| *H. neodissecta* | DQ279510 | EU562689 |
| *H. osseoalba* | DQ279512 | EU562690 |
| *H. physcioides* | DQ279515 | JN939618 |
| *H. pseudosinuosa* | DQ279517 | EU562692 |
| *H. revoluta* | DQ279523 | AY607787 |
| *H. rockii* | DQ279524 | EU562693 |
| *H. sinuosa* | DQ279527 | AY607788 |
| *H. taylorensis* | DQ279528 | AY578924 |
| *M. fuliginosa* | AY611124 | AJ421428 |
| *M. glabra* | AY611114 | AJ421427 |
| *M. subargentifera* | AY611098 | AJ421429 |
| *M. subaurifera* | AY611118 | AJ421432 |

| | GenBank Acc. No. | |
|---|---|---|
| species | nuITS | nuLSU |
| *Melanohalea aff elegantula* | AY611120 | AY607834 |
| *M. aff exasperata* | AY611092 | AY607804 |
| *M. elegantula* | AY611094 | AJ421437 |
| *M. exasperata* | AY611083 | AJ421438 |
| *M. exasperatula* | AY611090 | AJ421436 |
| *M. olivacea* | AY611091 | AY607803 |
| *M. septentrionalis* | AY611093 | AY607805 |
| *M. subelegantula* | AY611115 | AY607829 |
| *M. subolivacea* | AY611123 | AY607837 |
| *Myelochroa irrugans* | DQ394385 | AY607815 |
| *M. metarevoluta* | AY611102 | AY607814 |
| *Parmelia discordans* | AY583212 | EF042918 |
| *P. saxatilis* | AF058037 | AY578947 |
| *P. saxatilis* | AF141370 | AY300849 |
| *P. serrana* | AF350031 | AY578948 |
| *P. squarrosa* | AY036979 | AY607816 |
| *P. sulcata* | AY580313 | AY578949 |
| *Parmelina carporrhizans* | DQ273854 | DQ268552 |
| *P. carporrhizans* | DQ273853 | DQ268551 |
| *P. coleae* | DQ273856 | DQ268554 |
| *P. coleae* | DQ273855 | DQ268553 |
| *P. pastillifera* | AY611104 | AY607817 |
| *P. quercina* | DQ273848 | DQ268546 |
| *P. tiliacea* | AY581084 | AY578950 |
| *Parmelinella wallichiana* | AY611106 | AY607819 |
| *Parmelinopsis cryptochlora* | DQ279535 | EU562695 |
| *P. horrescens* | DQ279537 | AY578951 |
| *P. minarum* | DQ279539 | AY578952 |
| *P. neodamaziana* | AY611107 | AY607820 |
| *P. subfatiscens* | AY611108 | AY607821 |
| *Parmeliopsis ambigua* | AF451764 | AY607822 |
| *P. hyperopta* | AY611109 | AY607823 |
| *Parmotrema cetratum* | AY586576 | AY584847 |
| *P. crinitum* | AY586565 | AY584837 |
| *P. fistulatum* | AY581057 | AY578920 |
| *P. haitiense* | AY581055 | AY578918 |
| *P. hypoleucinum* | AY586567 | AY584839 |
| *P. perforatum* | AY586568 | AY584840 |
| *P. perlatum* | AY586566 | AY584838 |
| *P. pilosum* | AY581056 | AY578919 |
| *P. reticulatum* | AY586577 | AY584848 |
| *P. robustum* | AY586569 | AY584841 |
| *P. subcaperatum* | AY586557 | AY584829 |
| *P. subthomsonii* | AY586564 | AY584836 |
| *P. subtinctorium* | AY586558 | AY584830 |
| *P. tinctorum* | AB177404 | AY584635 |
| *Pleurosticta acetabulum* | AY581087 | AY578953 |
| *Punctelia borreri* | DQ394373 | AY578954 |
| *P. jeckeri* | AY613407 | GU994625 |
| *P. perreticulata* | AY773124 | GU994626 |
| *P. pseudocoralloidea* | AY586572 | AY584843 |
| *P. reddenda* | AY613410 | GU994627 |

| | GenBank Acc. No. | |
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| species | nuITS | nuLSU |
| *P. rudecta* | AY586574 | AY584636 |
| *P. sp.* | GU994579 | GU994628 |
| *P. subflava* | AY586575 | AY584846 |
| *P. subrudecta* | AY773118 | AY578955 |
| *Remototrachyna adducta* | DQ279492 | AY785263 |
| *R. aff brevirhiza* | DQ279494 | - |
| *R. ciliata* | AY785273 | AY785266 |
| *R. costaricensis* | AY785269 | AY785262 |
| *R. crenata* | DQ279495 | EU562683 |
| *R. flexilis* | DQ279500 | EU562685 |
| *R. incognita* | DQ279507 | EU562687 |
| *R. infirma* | DQ279508 | AY785264 |
| *R. koyaensis* | DQ279509 | EU562688 |
| *R. scytophylla* | DQ279525 | EU562694 |
| *Usnea antarctica* | DQ219311 | DQ883692 |
| *U. florida* | AF117996 | EF113545 |
| *U. trachycarpa* | AJ748103 | AJ748103 |
| *Xanthoparmelia aff. epacridea* | HM125752 | HM125777 |
| *X. aff. supposita* | HM125749 | HM125773 |
| *X. aff. supposita* | HM125751 | HM125775 |
| *X. aliphaticella* | **JQ912377** | **JQ912470** |
| *X. aliphaticella* | **JQ912379** | **JQ912472** |
| *X. angustiphylla* | AY581092 | AY578958 |
| *X. atticoides* | AY581066 | AY578929 |
| *X. azaniensis* | EF042900 | EF042910 |
| *X. bibax* | **JQ912373** | **-** |
| *X. brachinaensis* | AY581062 | AY578925 |
| *X. chalybaeizans* | GU903332 | GU903341 |
| *X. competita* | **JQ912383** | **JQ912476** |
| *X. condyloides* | HM125754 | - |
| *X. condyloides* | **JQ912374** | **JQ912468** |
| *X. conspersa* | AY581096 | AY578962 |
| *X. cordillerana* | **JQ912358** | **JQ912453** |
| *X. crespoae* | AY581097 | AY578963 |
| *X. digitiformis* | AY581099 | AY578965 |
| *X. exornata* | EF042908 | EF108318 |
| *X. greytonensis* | HM125755 | HM125780 |
| *X. hottentota* | EF042909 | EF042919 |
| *X. hueana* | AY581090 | AY578956 |
| *X. hypoprotocetrarica* | **JQ912378** | **JQ912471** |
| *X. hypoprotocetrarica* | **JQ912380** | **JQ912473** |
| *X. ianthina* | **JQ912360** | **JQ912455** |
| *X. isidiovagans* | AY581094 | AY578960 |
| *X. lineola* | - | AY578970 |
| *X. lithophila* | AY581077 | AY578941 |
| *X. lithophiloides* | AY581078 | AY578942 |
| *X. mexicana* | **JQ912354** | **JQ912451** |
| *X. mexicana* | **JQ912386** | **JQ912479** |
| *X. murina* | AY581079 | AY578943 |
| *X. neoquintaria* | **JQ912382** | **JQ912475** |
| *X. neotumidosa* | HM125758 | HM125782 |
| *X. norcapnodes* | AY581080 | AY578944 |

| | GenBank Acc. No. | |
|---|---|---|
| species | nuITS | nuLSU |
| *X. notata* | AY581101 | AY578968 |
| *X. ovealmbornii* | EF042901 | EF042911 |
| *X. peltata* | DQ980021 | DQ923670 |
| *X. perezdepazzii* | **JQ912355** | **-** |
| *X. perspersa* | GU903338 | GU903347 |
| *X. perspersa* | GU992330 | HM125772 |
| *X. perspersa* | GU903339 | GU903348 |
| *X. perspersa* | GU992333 | HM125778 |
| *X. perspersa* | GU992329 | HM125769 |
| *X. perspersa* | HM125758 | HM125783 |
| *X. perspersa* | GU992328 | HM125762 |
| *X. perspersa* | GU992332 | HM125776 |
| *X. protomatrae* | AY581104 | AY578972 |
| *X. protomatrae* | **JQ912376** | **JQ912469** |
| *X. ralla* | GU903337 | GU903346 |
| *X. reptans* | AY581102 | AY578969 |
| *X. saxeti* | AY581063 | AY578926 |
| *X. saxeti* | HM125739 | HM125760 |
| *X. scitula* | GU903333 | GU903342 |
| *X. scitula* | HM125741 | HM125763 |
| *X. scitula* | GU903334 | GU903343 |
| *X. scotophylla* | AY581081 | AY578945 |
| *X. semiviridis* | AY581058 | AY578921 |
| *X. sigillata* | **JQ912359** | **JQ912454** |
| *X. sp.* | **JQ912363** | **JQ912458** |
| *X. sp.* | **JQ912367** | **JQ912462** |
| *X. sp.* | **JQ912357** | **-** |
| *X. sp.* | **JQ912361** | **JQ912456** |
| *X. sp.* | **JQ912366** | **JQ912461** |
| *X. sp.* | **JQ912369** | **JQ912464** |
| *X. sp.* | **JQ912375** | **-** |
| *X. stenophylla* | AY581093 | AY578959 |
| *X. stenophylla* | **JQ912370** | **JQ912465** |
| *X. stenophylla* | **JQ912372** | **JQ912467** |
| *X. subamplexuloides* | HM125753 | HM125779 |
| *X. subchalybaeizans* | **JQ912385** | **JQ912478** |
| *X. subchalybaeizans* | GU903336 | GU993345 |
| *X. subchalybaeizans* | HM125750 | HM125774 |
| *X. subchalybaeizans* | HM125743 | HM125765 |
| *X. subchalybaeizans* | GU903331 | GU903340 |
| *X. subchalybaeizans* | HM125759 | HM125784 |
| *X. subchalybaeizans* | HM125747 | HM125770 |
| *X. subchalybaeizans* | HM125745 | HM125767 |
| *X. subchalybaeizans* | HM125748 | HM125771 |
| *X. subdiffluens* | AY581105 | AY578973 |
| *X. subdiffluens* | **JQ912381** | **JQ912474** |
| *X. subdomokosii* | **JQ912384** | **JQ912477** |
| *X. sublaevis* | AY581106 | AY578974 |
| *X. sublaevis* | **JQ912356** | **JQ912452** |
| *X. subpodochroa* | AY581082 | AY578946 |
| *X. subverrucigera* | AY581091 | AY578957 |
| *X. tentaculina* | HM125756 | HM125781 |

| | GenBank Acc. No. | |
|---|---|---|
| species | nuITS | nuLSU |
| *X. tinctina* | **JQ912365** | **JQ912460** |
| *X. tinctina* | **JQ912368** | **JQ912463** |
| *X. tinctina* | **JQ912371** | **JQ912466** |
| *X. tinctina* | AY581108 | AY578976 |
| *X. tinctina* | AY581109 | AY578977 |
| *X. tortula* | HM125742 | HM125764 |
| *X. transvaalensis* | AY581095 | AY578961 |
| *X. tzaneenensis* | **JQ912364** | **JQ912459** |
| *X. verrucigera* | **JQ912362** | **JQ912457** |
| *X. verrucigera* | AY581111 | AY578979 |
| *X. vicentei* | AY581112 | AY578980 |

## Discusión general

El género *Xanthoparmelia* fue propuesto para segregar del género colectivo *Parmelia* las especies saxícolas que presentan ácido úsnico en el córtex, ricinas simples en la cara inferior y carecen de cilios (Hale, 1974). En los dos primeros capítulos de esta tesis doctoral hemos profundizado en la caracterización del género *Xanthoparmelia* al estudiar la posición filogenética y los caracteres fenotípicos de tres géneros próximos: *Karoowia*, *Placoparmelia* y *Omphalodiella*. El género *Karoowia* separó de *Xanthoparmelia* un conjunto homogéneo de 16 especies con biotipo subcrustáceo, rizoides en la cara inferior y conidios cilíndricos (Hale, 1989). *Omphalodiella* y *Placoparmelia* fueron descritos en base a sus diferencias morfológicas en la anatomía del ascoma y en la forma de crecimiento del talo o biotipo (Hessen, 1991, 1992).

A través del estudio de varios marcadores moleculares de ADN hemos comprobado que *Karoowia* es polifilético dentro del clado 'xanthoparmelia' (Figura 1, Artículo 2) y hemos detectado que presenta al menos 7 linajes independientes entre si. Los géneros *Placoparmelia* y *Omphalodiella* son monoespecíficos, tienen una distribución geográfica restringida y ambos resultan ser linajes internos dentro del clado 'xanthoparmelia' (Figura 1, Artículo 1). *Placoparmelia patagonica* parece relacionarse con el grupo '*Xanthoparmelia pulla*', aunque su relación filogenética no presenta suficiente apoyo estadístico (Artículo 1). *Omphalodiella patagonica* forma parte del linaje constituido por las especies del antiguo género *Almbornia* (Thell *et al*., 2006), que se describió también en base a una peculiar forma de crecimiento del talo (Esslinger, 1981), junto con otras especies subcrustáceas del género *Xanthoparmelia* con o sin ácido úsnico en el córtex (Figura 1, Artículo 2).

Hasta el momento se han encontrado dos caracteres diagnósticos propios del clado 'xanthoparmelia': el polisacárido de pared celular tipo 'Xanthoparmelia liquenana' (Blanco *et al*., 2004b) y la presencia de un cuerpo vacuolar araquiforme en la ascóspora (Del Prado *et al.,* 2007). Estos caracteres están presentes en las especies estudiadas de los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia*, por lo que podemos ratificar su correlación con este clado.

Se ha especulado si la presencia del cuerpo vacuolar arachiforme en la ascóspora es el carácter que ha permitido la adaptación a la vida en ambientes terrícolas áridos y el éxito de este linaje. Apoya esta hipótesis el hecho de que en *Xanthoparmelia* el porcentaje de especies que presentan ascomas frente a las que se multiplican únicamente por vía asexual es mucho mayor que en otros grupos de parmelioides (Del Prado *et al.*,

2007). El otro carácter diagnóstico (la presencia del tipo peculiar de polisacárido de pared celular) también podría estar ligado a la adaptación y éxito frente a la vida en ambientes áridos. Trabajos realizados en plantas vasculares muestran que los polisacáridos de la pared celular están implicados en las estrategias de tolerancia al estrés hídrico y a la desecación (Leucci *et al*., 2008; Moore *et al.*, 2008). En hongos liquenizados el valor de adaptación ecofisiológico frente al estrés hídrico del polisacárido de la pared celular aún no ha sido estudiado pero en el caso de *Xanthoparmelia* podría ser un factor determinante de su éxito y su enorme radiación al colonizar ambientes saxícolas y terrícolas con estrés hídrico.

Los caracteres fenotípicos en los que se basó la descripción de los géneros *Karoowia*, *Placoparmelia* y *Omphalodiella*, casi todos relacionados con la forma de crecimiento del talo, también se han reexaminado en base a los resultados de los marcadores moleculares. En *Xanthoparmelia* la forma de crecimiento del talo más frecuente es el biotipo foliáceo, sin embargo existe una significativa variabilidad desde los talos umbilicados de *Xanthoparmelia hottentotta* (= *Xanthomaculina hottentotta*) o *Xanthoparmelia nautilomontana* (= *Neofuscelia nautilomontana*), pasando por los talos fruticulosos de *Xanthoparmelia azaniensis* (= *Almbornia azaniensis*) o *Xanthoparmelia lichinoidea* (= *Neofuscelia lichinoidea*) a los talos subcrustáceos de *Xanthoparmelia mougeotina* o *Xanthoparmelia parviloba* (= *Neofuscelia parviloba*).

Las especies de *Karoowia* y *Placoparmelia* (con talos subcrustáceo y placoideo, respectivamente) representarían el máximo grado de unión entre el talo y el sustrato en el clado 'xanthoparmelia' dentro de la variabilidad de formas de crecimiento de este linaje. De esta estrecha unión del talo al sustrato deriva que el córtex inferior y las rizinas estén muy reducidas (en *Karoowia*), o sean casi inapreciables (en *Placoparmelia*). La estrecha unión con el sustrato está relacionada con la adaptación de las especies a vivir en situaciones muy expuestas a los factores ambientales (Büdel & Scheidegger, 2008). El biotipo escuamuloso-peltado de *Omphalodiella patagonica* si parece representar un cambio cualitativo en la forma de crecimiento del talo, ya que este biotipo no aparece en ninguna otra especie del clado; este biotipo aparece en especies adaptadas a vivir sobre rocas o suelo en zonas cálidas y secas del planeta (Büdel & Scheidegger, 2008).

La variabilidad y capacidad rápida de adaptación que presenta la forma de crecimiento del talo es enorme, existiendo un gran número de convergencias o paralelismos entre los diferentes grupos evolutivos de hongos liquenizados (Grube & Hawsworth, 2007). Según nuestro estudio, los caracteres vegetativos que están directamente asociados a la

forma de crecimiento, como el tamaño de las ricinas y el grosor del córtex inferior, también presentan esta variabilidad y capacidad de adaptación. Como casos extremos de esta variabilidad tenemos el ejemplo de la plasticidad de los fotomorfos, en el que una misma especie de hongo adquiere una forma del talo u otra en función del tipo de fotobionte (Armaleo & Clerc, 1991). En el clado 'xanthoparmelia' también hay un interesante ejemplo de plasticidad de la forma de crecimiento del talo a nivel de especie. El biotipo pulvinado-vagante, que ha sido usado como carácter taxonómico a nivel de especie en *Xanthoparmelia*, no se correlaciona con la filogenia basada en caracteres moleculares y parece ser una adaptación facultativa que pueden presentar varias especies, según un trabajo de poblaciones reciente en especies del Oeste de Norteamérica (Leavitt *et al.*, 2011a). Esto no quiere decir que sea un carácter facultativo en todos los casos, puede haber especies que presentan este biotipo de forma constante. En el estudio llevado a cabo en el grupo '*Xanthoparmelia pulla'* los datos moleculares no son del todo concluyentes respecto al valor de la forma de crecimiento del talo como carácter taxonómico diferencial entre las especies *X. pokornyi* (pulvinado-vagante) y *X. pulla* (foliáceo). En nuestra observación de campo hemos encontrado algunos especímenes intermedios, en los que la mitad del talo era pulvinado y la otra mitad foliáceo, lo que apunta a que en este caso también se trate de un carácter facultativo.

Al igual que en la forma de crecimiento del talo, las convergencias y los paralelismos en el desarrollo de los diferentes tipos de ascomas son frecuentes en los hongos liquenizados (Grube & Hawsworth, 2007; Rivas-Plata & Lumbsch, 2011). La filogenia molecular de *Xanthoparmelia* muestra ejemplos de probable paralelismo en la anatomía del apotecio. *Omphalodiella* y *Placoparmelia* presentan similitudes en el desarrollo del ascoma: ambas especies tienen apotecios zeorinos con la cúpula parmelioide no estructurada en capas. Esto llevó a plantear la posibilidad de que ambos géneros constituyeran un linaje evolutivo de la familia Parmeliaceae en Sudamérica (Hessen, (1992). Según nuestra hipótesis, estas similitudes en el ascoma es probable que sean debidas al desarrollo aspicilioide del apotecio que presentan ambas especies (aunque en *Placoparmelia* solo se da en los primeros estadíos). Cuando en *Placoparmelia* el apotecio está plenamente desarrollado emerge sobre la superficie del talo, pasando a tener una disposición "sentada". En está posición el subhimenio aparece hialino, diferenciándose de la cúpula parmelioide, y el excípulo propio queda envuelto entre el himenio y el excípulo talino, desapareciendo la disposición de los excípulos en forma de apotecio zeorino que se observaba en las primeras fases de desarrollo (Figura 2F, Artículo 1). Otras especies subcrustáceas de *Karoowia* y *Xanthoparmelia* también presentan un desarrollo de los apotecios de tipo aspicilioide en la etapas juveniles (Hale, 1989, 1990), por lo que este sería otro caso de un carácter que puede estar relacionado

con la forma de crecimiento del talo. En otros géneros de líquenes que presentan especies con un biotipo escuamuloso-peltado (similares a *Omphalodiella*) también son frecuentes los apotecios aspicilioides. En otras especies del clado 'xanthoparmelia' en los que se han observado apotecios juveniles aspicilioides con aspecto zeorino, es frecuente observar también apotecios maduros de claro aspecto lecanorino, por lo que es posible que este carácter de la ontogenia del apotecio haya pasado desapercibido.

La forma cilíndrica de los conidios de las especies que se incluyeron en *Karoowia*, frente a los bifusiformes a baciliformes de *Xanthoparmelia,* parece ser un carácter variable a lo largo del árbol filogenético del clado 'xanthoparmelia'. Este carácter no se encuentra ligado a la forma de crecimiento subcrustáceo del talo, ya que hay especies de *Xanthoparmelia* con talo foliáceo y conidios cilíndricos e incluso algunas especies de *Karoowia* presentan conidios bifusiformes (Figura 4C,D; Artículo 2). Al igual que el tipo de crecimiento subcrustáceo del talo, la forma de los conidios es un carácter polifilético, no muestra una señal evolutiva clara entre los principales linajes internos ni en las ramas terminales del clado *Xanthoparmelia*.

Según la filogenia molecular y la reinterpretación de los caracteres morfológicos hemos propuesto sinonimizar los géneros *Karoowia*, *Placoparmelia* y *Omphalodiella* dentro del género *Xanthoparmelia*, dando de esta manera el rango de género (*Xanthoparmelia*) a todo el linaje evolutivo que hasta ahora hemos denominado como clado 'xanthoparmelia'.

Tras abordar el primer objetivo de esta tesis doctoral se estudiaron las especies de amplia distribución del grupo '*Xanthoparmelia pulla*'. Los primeros resultados mostraron la polifilia de estas especies y la correlación de los linajes con el área geográfica de recolección de los especímenes, llamando sobre todo la atención la relación filogenética entre las especies sudamericanas y australianas. Ante este patrón filogeográfico observado en el grupo '*X. pulla*', la estimación de la edad de los linajes resultaba un dato clave para entender la historia evolutiva de estas especies, esclarecer su patrón geográfico y estudiar el papel de la vicarianza y la dispersión a larga distancia en estos hongos liquenizados. Por tanto un objetivo fundamental fue estimar la edad del género y sus principales linajes. Debido a la ausencia de registro fósil en el género *Xanthoparmelia*, hubo que realizar una calibración combinada de todo el linaje parmelioide. Se usaron tres puntos de calibración: uno en un nodo próximo a la raíz del árbol y otros dos en nodos internos, uno de ellos dentro del linaje parmelioide, lo que confería una mayor estabilidad al análisis (Ho *et al.* 2009; Forest, 2009).

De esta forma, en el capítulo tercero hemos realizado la primera filogenia datada de los líquenes parmelioides, estimando la edad de divergencia de los linajes resueltos (con soporte estadístico) y la edad de radiación de los principales géneros. Al usar un árbol filogenético que parte del origen de Lecanoromycetes, también se obtuvo una estimación de la edad de otros clados dentro de esta Clase. Aunque nuestro muestreo era poco representativo en las ramas terminales para los grupos no parmelioides, los datos obtenidos para estos linajes concuerdan con los obtenidos en trabajos previos. Así, Berbee & Taylor (2010) calcularon la edad de diversificación de la familia Parmeliaceae usando la mayor distancia genética encontrada en el marcador nuSSU entre dos especies de la familia (*Menegazzia terebrata* y *Parmelia saxatilis*) y diferentes tasas de mutación: su resultado fue que la radiación debió producirse antes de la edad mínima que se disponía para la familia (40 Ma), apoyada por el fósil más antiguo (Rikkinen & Poinar, 2002). Berbee & Taylor (2010) dieron una edad entre los 62 - 100 Ma y nuestros resultados indicaron 74 Ma (57 – 92). Teniendo en cuenta que entre la aproximación de Berbee & Taylor (2010) y la nuestra los datos de partida y los métodos eran muy diferentes, la similitud entre los resultados refuerza la validez del análisis. En cuanto al fósil interno de la familia Parmeliaceae, en nuestro análisis hemos utilizado un fósil de edad similar al indicado por Berbee & Taylor (2010), ya que el fósil de *Anzia electra* (Rikkinen & Poinar, 2002) y el de *Alectoria succini* (Mägdefrau, 1957) se encuentran en el mismo tipo de ambar y tienen, por tanto, una edad similar.

La estimación de la edad de los linajes en el árbol filogenético parmelioide nos ha permitido asignar un mayor papel a la dispersión a larga distancia en la explicación de las áreas de distribución disyunta actuales, principalmente en los grupos de especies del Hemisferio Sur, ya que el inicio de la fractura de Gondwana en las diferentes masas continentales se produjo mucho antes (135 Ma, Sanmartín & Ronquist, 2004) que el origen de los linajes que actualmente están distribuidos en estas regiones (47- 29 Ma). Según la comparación de las similitudes florísticas de musgos, hepáticas, líquenes y helechos en el Hemisferio Sur, las "autopistas de viento" (corrientes de aire de dirección constante) son el vehículo de dispersión a larga distancia entre las masas terrestres que rodean el polo sur (Muñoz *et al.*, 2004). A pesar de ello, nuestros resultados no permiten descartar la vicarianza debida a deriva continental para las disyunciones entre Sudamérica y Australia (edad de separación continental: 52-35 Ma, Sanmartín & Ronquist, 2004), al solaparse esta edad con la edad de los linajes.

La reconstrucción de una filogenia general datada de los parmelioides nos ha permitido además relacionar las condiciones paleoclimáticas de la Tierra en las que se produjo la diferenciación con la radiación de los principales linajes. La gran mayoría de los

géneros comenzaron su radiación durante el Mioceno, época geológica en que las condiciones generales climáticas del planeta comenzaron a ser más frías, secas y estacionales (Zachos *et al*. 2008).

El estudio de la filogenia del grupo '*Xanthoparmelia pulla*' se centró principalmente en las especies de amplia distribución. Este análisis no respalda el concepto fenotípico (morfoquímico) de estas especies, sino que muestra un patrón de correlación con las áreas geográficas de los especímenes y con la composición de metabolitos secundarios medulares (extrolitos) (Figuras 1 y 2, Artículo 4). Nuestros resultados respaldan la necesidad de una revisión del concepto de especie en este género debido a que la gran plasticidad de los caracteres fenotípicos los invalida en muchos casos para el reconocimiento de los diferentes linajes (Lumbsch & Leavitt, 2011).

Nuestros datos nos permiten extraer algunas conclusiones sobre el origen y la dispersión del grupo '*X. pulla*' sobre todo en relación a Sudáfrica y Australia. Es en estas dos regiones donde tanto el conjunto del género *Xanthoparmelia* como el grupo '*X. pulla*' presenta una mayor riqueza de especies fenotípicas (Introducción, Figura 1).

Partiendo de los resultados del artículo 3, hemos realizado una datación del grupo '*X. pulla*', un análisis filogeográfico y una reconstrucción del área ancestral (Artículo 4). Los resultados señalan a Sudáfrica como la región con la mayor probabilidad de ser el centro de diversificación del grupo, con una edad de *ca.* 11 Ma (7.61 – 16.50) para el inicio de esta diversificación. Esta edad coincide con el cambio climático debido a modificaciones en las corrientes oceánicas que extendió la aridez en la zona oeste de Sudáfrica (Richardson *et al.*, 2001). El origen de la riqueza en flora vascular de la Región del Cabo ha sido relacionada con esta extensión de la aridez, junto con otros factores como la alta diversidad de tipos de suelos, la compleja geografía y bioclimatología, el aislamiento de poblaciones producido por la frecuencia de fuegos, cambios en los polinizadores (Richardson *et al.*, 2001), y cierta estabilidad climática a partir de la llegada de la aridez (Schnitzler *et al.*, 2011). Según nuestros resultados, el grupo '*X. pulla*' podría tener el mismo origen temporal y espacial que la flora vascular de la Región del Cabo. Muchos de los factores ambientales relacionados con la riqueza en flora vascular han podido contribuir también a generar la diversidad genética que muestra el grupo '*X. pulla*' en esta región.

A partir de este centro de origen sudafricano, el grupo '*X. pulla*' se ha dispersado a otras regiones y ha sufrido radiaciones sucesivas. Este hecho pone de manifiesto la compatibilidad de la dispersión a larga distancia con el aislamiento entre regiones

geográficas, lo que tiene importantes consecuencias de cara a la conservación de la biodiversidad de estos organismos. Este patrón de dispersión/especiación, junto con la direccionalidad de la dispersión (Muñoz *et al.*, 2004; Sanmartin *et al*., 2007) pone límites a la hipótesis microbiológica de "todo está en todos los sitios" (Finlay, 2002) en el grupo '*X. pulla*', al existir un aislamiento entre regiones geográficas que parece ser roto por fenómenos puntuales de dispersión a larga distancia (Nathan, 2006). Según el momento en que se produjo la conexión entre las áreas podemos distinguir, a grandes rasgos, dos patrones:

- Fenómenos puntuales de dispersión a larga distancia antiguos, seguidos de aislamiento. Han dado pie a nuevas radiaciones. En el grupo '*X. pulla*' serían los casos de formación del clado 1 en el Hemisferio Norte, el clado 2 en Australia, clado 2.2 en Sudamérica, clado 4 en América (Figuras 1 y 2, Artículo 4). Estos resultados son coherentes con los trabajos que muestran un patrón entre la filogenia de los grupos y la geografía (ejemplos de este tipo existen en *Parmelina* (Arguello *et al.*, 2007), *Leptogium* (Otálora *et al.*, 2010), *Austroparmelina* (Crespo *et al*., 2010) y *Nephroma* (Sérusiaux *et al.*, 2011).

- Fenómenos puntales de dispersión a larga distancia recientes o que se producen con cierta frecuencia entre determinadas áreas. Serían los casos de la conexión entre Norteamérica y la cuenca mediterránea (interior del clado 1) o la conexión entre Norteamérica y Sudamérica (interior del clado 4) en el grupo '*X. pulla*' (Figuras 1 y 2, Artículo 4). En los casos en los que se ha producido una dispersión puntual reciente, puede que los marcadores moleculares no hayan tenido suficiente tiempo de acumular mutaciones como para detectarlas en el análisis filogenético o que exista un cierto flujo de migrantes que impide el aislamiento y la deriva génica. Ejemplos de este comportamiento existen en *Cladonia* (Myllys *et al.*, 2003), *Parmelia* (Molina *et al.*, 2004) y *Porpidia* (Buschbom, 2007).

Los datos moleculares también muestran que la química medular puede reflejar una cierta señal filogenética: los especímenes del clado 4 presentan de forma constante ácido hipoestíctico, además de un patrón geográfico propio. Este síndrome químico en Sudamérica y en California sería un carácter indicativo para diferenciar entre los dos linajes detectados en cada una de las áreas. En el resto de linajes también existe la tendencia a conservar la ruta biosintética de formación de los metabolitos secundarios medulares (la ruta biosintética de derivados del orcinol es más rica en compuestos finales). Esta señal filogenética relacionada con la ruta biosintética de metabolitos había

sido puesta de manifiesto en otra familia de hongos liquenizados (Pertusariaceae, Schmitt & Lumbsch, 2000).

La información obtenida en este trabajo permitirá plantear un estudio de poblaciones incorporando diversos marcadores moleculares para la delimitación de especies dentro de los principales clados que presenta el grupo '*X. pulla*'.

## Conclusiones generales

Los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia* pertenecen al mismo linaje evolutivo que las especies del género *Xanthoparmelia* según los datos de marcadores moleculares de ADN. Los especímenes analizados de estos géneros presentan además el polisacárido de pared celular tipo 'Xanthoparmelia liquenana' y un cuerpo vacuolar araquiforme en la ascóspora, ambos caracteres diagnósticos del clado 'xanthoparmelia'.

Los caracteres fenotípicos en los que se basó la descripción de los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia* se incluyen dentro de la gran variabilidad morfológica que presenta en conjunto el género *Xanthoparmelia*. Sólo el biotipo escuamuloso-peltado de *Omphalodiella patagonica* presenta un novedoso carácter funcional dentro del clado 'xanthoparmelia', con una adaptación que se relaciona con el crecimiento sobre superficies expuestas al sol en zonas áridas.

Se ha realizado la sinonimia de los géneros *Karoowia*, *Omphalodiella* y *Placoparmelia* en *Xanthoparmelia*.

El origen de la diversificación de los líquenes parmelioides estimamos que se produjo en torno al tránsito entre el Cretácico y el Terciario, hace unos 60 Ma, mientras que la radiación de los diferentes géneros parmelioides ocurrió entre el Oligoceno y el Plioceno. Según esta estimación, la mayoría de las distribuciones actuales que presentan los líquenes parmelioides no pueden ser explicadas por vicarianza debida a la deriva continental, por lo que la dispersión a larga distancia a nivel global entre continentes ha jugado el papel principal en la distribución que presentan estos líquenes. Sin embargo, en la disyunción entre Sudamérica y Australia la edad de los linajes a nivel de género no permite descartar la vicarianza.

El género *Xanthoparmelia* estimamos que apareció durante el Eoceno por escisión del clado 'parmotrema' y la diversificación de los linajes hoy en día existentes comenzó entre el Oligoceno y el Mioceno.

El concepto morfoquímico de especie en el grupo *Xanthoparmelia pulla* no se corresponde con los linajes evolutivos de la filogenia basada en marcadores moleculares de ADN. Estos linajes tienen por el contrario una correspondencia con la distribución geográfica de las especies a nivel mundial y con el tipo de metabolitos secundarios medulares que presentan los talos.

Muy probablemente el grupo *Xanthoparmelia pulla* comenzó su radiación en Sudáfrica hace unos 11 Ma, coincidiendo con la aridificación de los ecosistemas de la zona, y desde este territorio se ha extendido por fenómenos de dispersión a larga distancia a otros continentes, donde ha sufrido posteriores radiaciones. Una de ellas, la que se produjo en Australia, se ha dispersado secundariamente hasta Sudamérica también por dispersión a larga distancia.

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